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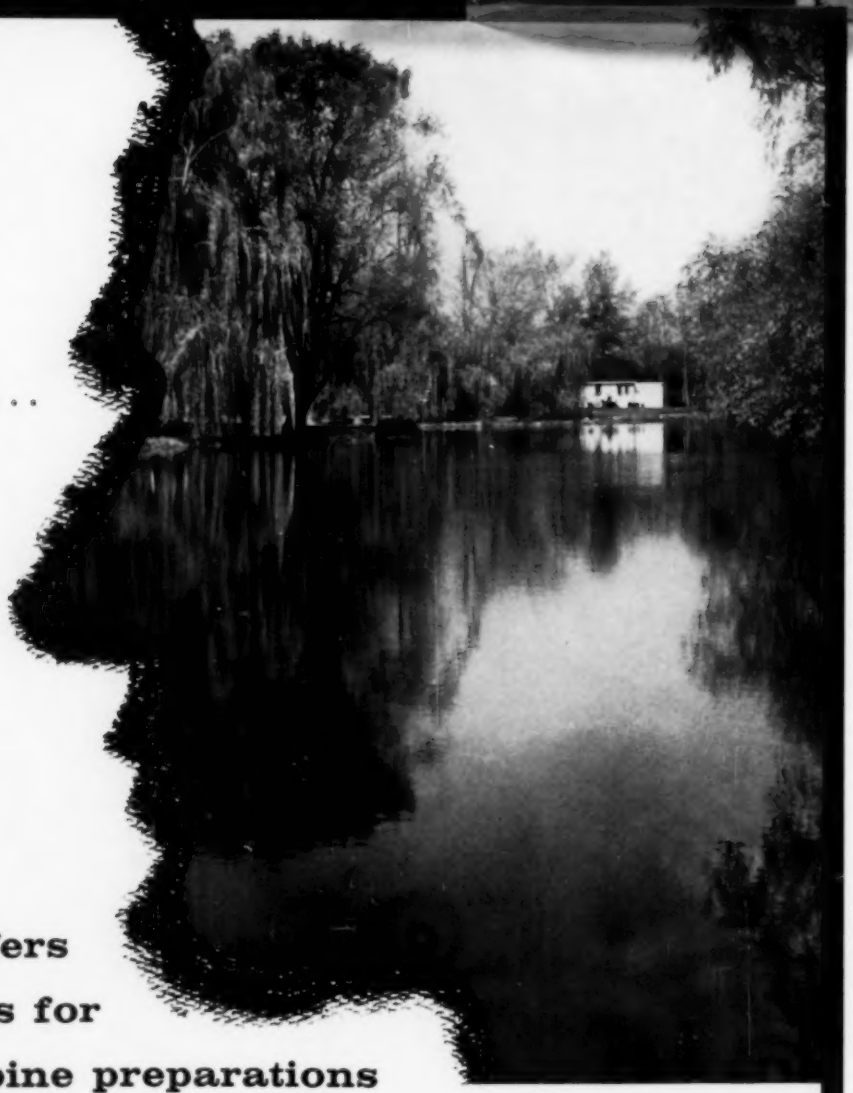

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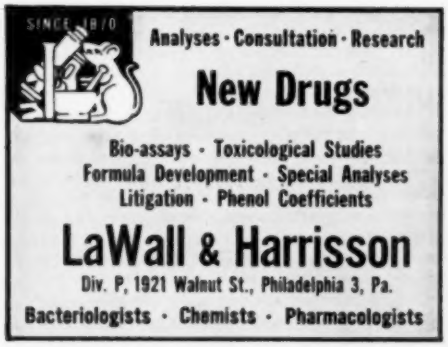
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FIBRINOGEN (HUMAN).—Parenogen (Cutter).—Fibrinogen is a protein essential for blood coagulation. It is prepared by alcohol fractionation from human plasma collected and prepared in accordance with the regulations of the National Institutes of Health.

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In the employment of fibrinogen (human) the physician should weigh very carefully the hazard of transmitting the virus of serum hepatitis against the danger of hemorrhagic complications in which it may be lifesaving. It should not be employed in the absence of adequate blood coagulation studies and determination of plasma fibrinogen levels, except in emergencies when facilities for performing these procedures are not available. A plasma fibrinogen level of less than 50 mg. per 100 cc. is considered critically low; levels below 100 mg. per 100 cc. may indicate the need for fibrinogen, but, in chronic hypofibrinogenemia, low levels may be encountered without frank hemorrhage.

Dosage.—Fibrinogen (human) is administered intravenously after reconstitution of a dried preparation with water for injection; 1 Gm. of dried fibrinogen (human) is dissolved in 50 cc. of pyrogen-free sterile distilled water. The dried product should be stored at 2 to 10°. It should be administered within one hour after reconstitution. In acute afibrinogenemia associated with premature separation of the placenta, the usual dosage is 2 to 6 Gm. (100 to 300 cc.), depending upon the response of the patient. In other afibrinogenemic or hypofibrinogenemic conditions, the dosage should be adjusted according to the clinical observations and initial plasma fibrinogen levels. Whenever feasible, the latter are invaluable as an aid for determining the adequacy of the dosage.

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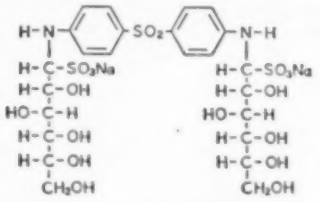

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The drug is effective in the treatment of leprosy. Against tuberculosis the results have been less dramatic, although beneficial effects have been reported. Streptomycin is much more effective in tuberculosis than is glucosulfone sodium, but, when the two are given together, development of resistance to streptomycin is delayed; hence in tuberculosis glucosulfone sodium is used principally as an adjunct to streptomycin therapy.

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Dosage.—In the treatment of leprosy, the intravenous dose is 2 to 5 Gm. It is administered daily for six consecutive days, omitted on the seventh day, and administered daily for the next six days. Treatment is interrupted for one week at the conclusion of each two week period. This schedule should be maintained for many months, the precise duration depending on the patient's response. In the treatment of tuberculosis, glucosulfone sodium may be used according to the same dosage schedule in conjunction with the usual course of streptomycin therapy.

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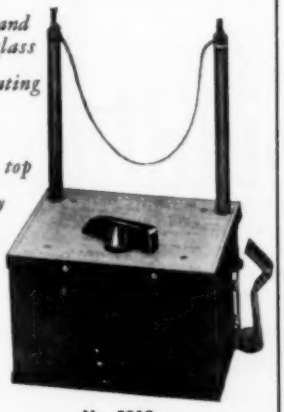

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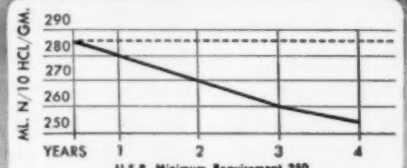

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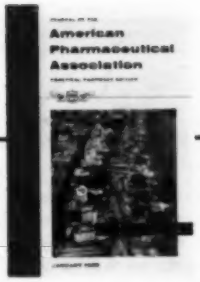


A Preview of Advertising

In the March 1956 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ Reserved by **Abbott Laboratories** are its familiar second cover and colorful inside spread to delineate the efficacy of **Iberol,** the "2-a-day therapy for the anemias," and the usefulness of its antibiotic, **Erythrocin.**

▸ For a ready-reference listing of pharmaceutical products, *arranged* according to therapeutic classifications and kept current by monthly revisions, readers indorse **"Facts and Comparisons."**

▸ In introducing **Clinitest,** a *new* urine-sugar test, **Ames** emphasizes its "utmost simplicity and convenience, complete specificity, extreme sensitivity, qualitative accuracy."

▸ For urinary infections, **Hoffmann-La-Roche** announces a new tablet, **Azo Gantrisin,** the virtue of which lies in the high plasma levels it provides.

▸ Indicated for their usefulness in the treatment of diarrhea are **Lactinex** *Granules* and *Tablets,* offered by **Hynson, Westcott & Dunning.**

▸ *New* from the laboratories of **Lederle** is **Achrocidin** "for the prevention and treatment of those conditions frequently associated with ordinary colds."

▸ "For a really vigorous vitamin regimen," **Eli Lilly and Company** reports that "physicians specify **Theracebrin** *Gelseals.*"

▸ Offered by **The National Drug Company** are the following "Products of Original Research:" **Parenzyme, Hesper-C, Hesper-C Liquid, AVC, Protinal, Vi-Protinal, Resion** and **Resion P-M-S.**

▸ To help the pharmacist's public relations, **Owens-Illinois** has made available a supply of leaflets instructing customers on how to store and safeguard the prescriptions they have filled.

▸ **Siblin,** product of **Parke, Davis & Company,** has as its purpose the reestablishment of normal bowel function in a variety of clinical conditions.

▸ **Pfizer's** brand of tetracycline, **Tetrabon,** and **Tetrabon SF** (tetracycline-hydrochloride with vitamins)—both homogenized—constitute flavorable mixtures of this eminent broad-spectrum antibiotic.

▸ **Pictorial Paper Package Corp.** is offering a *New Hypo Record* to satisfy all legal requirements covering the sale of hypodermic needles and syringes.

▸ Enumerating seven of its popular products, **A. H. Robins Co.** points up the *preselling* assistance rendered by its detailmen.

▸ **Schering's Coricidin,** *a name synonymous with cold control,* "confines the cold without confining the patient."

▸ The efficacy of **'Tetrazets',** reports **Sharp & Dohme,** is due to its *broader* attack to overcome minor throat irritations.

▸ Further testimony is offered by **Smith, Kline & French** to the sales-effectiveness of its **Benzedrex** inhaler display carton.

▸ Announcement is made by **Squibb** that **Mycostatin,** its antifungal antibiotic, is now available in the form of vaginal tablets, ointment, and oral tablets.

▸ Subject of **The Upjohn Company's** announcement is **Cheracol,** the usefulness of which is widely recognized in the treatment of coughs.

▸ **Peritrate** with *Aminophylline,* product of **Warner-Chilcott,** is indicated "especially in the presence of cardiac and circulatory inadequacy."

▸ The announcement made by **Winthrop Laboratories** emphasizes the profits to be realized from its product, **Neo-Synephrine.**

▸ "*Oral* penicillin with *injection* performance," is the descriptive phrase with which **Wyeth** tags **Pen Vee Oral.**

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out March 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLV — *MARCH 1956* — NUMBER 3 — CONSECUTIVE NO. 6

Photolysis of Vitamin B_{12}*

By LEON J. DeMERRE† and CATHERINE WILSON

The rate of destruction of crystalline vitamin B_{12} in neutral aqueous solutions, when exposed to direct sunlight, indirect sunlight, dim daylight, artificial light, ultraviolet light, and various monochromatic light sources has been determined. Sunlight, at a brightness of 8,000 foot-candles, causes a ten per cent loss for each half hour of exposure. Below 300 foot-candles, no destruction is noticeable. Artificial light of about 14,000 foot-candles causes a loss of about twelve per cent per half hour. A brightness of 3,600 foot-candles causes no alteration after two hours. With ultraviolet light the pattern of destruction is similar to that obtained with sunlight. For monochromatic light sources, photolysis is higher in the short wave length region. No destruction was observed in the red.

THE STABILITY of vitamin B_{12} has been studied in connection with reducing agents and heating conditions (1). Some information regarding the stability of this vitamin to light has also been reported (2). Yet, to our knowledge, no systematic observations of the latter type were ever made.

The present study deals with the rate of destruction of aqueous solutions of crystalline vitamin B_{12} submitted to various types of irradiation. The solutions were exposed to direct sunlight, dim daylight, artificial light of different intensities, as well as to rays in the visible and invisible spectra. The investigation was limited to solutions having a concentration of 10 micrograms per milliliter, the most common concentration used in pharmaceutical preparations (3*a*). The general procedure used at the present time in this series of experiments is essentially the same as that used in earlier work of this type (4, 5).

* Received July 13, 1955, from the Medical Laboratories, Army Chemical Center, Md.

† Present address: Stanford Research Institute, Menlo Park, Calif.

The authors are grateful to Dr. O. L. Kline, Dr. H. W. Loy, Jr., and to Mr. Parrish (Division of Nutrition, Food and Drug Administration, Washington, D. C.) for their assistance during the course of these experiments.

PROCEDURE

Preparation of Solutions.—A stock solution of cyanocobalamin titrating approximately 11 μg./ml. was prepared using double distilled water. It was distributed in 50-ml. glass stoppered Erlenmeyer flasks and autoclaved for 15 minutes at 121°C. In order to avoid the use of cotton plugs, the flasks were mounted between two metal plates applying sufficient pressure to hold the stoppers in place. The *p*H of the solutions after autoclaving would vary from 6.8 to 7.0, depending on the commercial source of the vitamin. The stability assays showed that after three months storage in the refrigerator, the potency of the solutions as measured by the spectrophotometric method had remained unchanged. Consequently, it was not deemed necessary to acidify them nor to add any buffers nor stabilizing agents. Immediately prior to light treatment the particular solution used was adjusted to 10 μg./ml., using a careful method of dilution and checking the concentration spectrophotometrically.

Technique of Exposure.—Three and five tenths milliliters of the solution was placed in flat-bottom test tubes of 22-mm. inside diameter, cut at 35 mm. from the bottom. The height of the liquid in these cuvettes was about 10 mm. Thus filled to a fiducial mark, the cuvettes were weighed. Compensation for evaporation was made intermittently during exposure time by adding double distilled water up to the mark. The liquid level was never allowed to go down more than 1 mm. Upon termination of

exposure time, each cuvette was restored to its original weight by means of a Gram-atic balance and micropipet.

A single experiment comprised a set of four cuvettes marked 30, 60, 90, and 120, respectively. These numbers corresponded to the times of exposure in minutes. Each set was submitted to the same lighting conditions. In every case, the light was allowed to strike the surface of the solution perpendicularly and air was the only medium between the source of radiation and the surface of the solution. A control sample of the solution was kept at room temperature in a box insuring complete darkness, during the entire course of the experiment. The samples, as they came off after their individual time of exposure, were stored next to the original one. For each particular set of experiments, at the conclusion of the last exposure time, the four samples and the original were assayed by the two methods described in the U. S. Pharmacopeia: spectrophotometric assay (3*b*) and microbiological assay (3*c*). The latter procedure was slightly modified.

EQUIPMENT AND PRELIMINARY EXPERIMENTS

Spectrophotometric Method.—A Beckman Model DU spectrophotometer was used, and the concentration of the solution was directly obtained in μg./ml. by dividing the absorbancy reading in the 10-mm. cells at 361 mμ by 0.0207.

Microbiological Assay.—The only divergence from the method referred to above was that the experimental solution was free of additives such as buffers, sodium bisulfite or alcohol. The test organism used was *Lactobacillus leichmannii* #7830[1], and the basal medium an exact duplication of that described in (6).

Each series of assays included a standard curve established for concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 millimicrogram of vitamin B_{12}, respectively. After inoculation, the tubes were incubated at 37° for approximately 18 hours. Turbidity readings relative to an inoculated blank set at 100% transmittancy were made on a Beckman DU spectrophotometer at a wavelength of 550 mμ. In no case did the difference in transmittancy between the inoculated and the uninoculated blanks exceed 15%.

A standard curve taken at random is herewith presented (Fig. 1). It clearly indicates that the working part of the curve is to be found for concentrations from 0.00 to 0.03 millimicrogram. The curve tends toward a plateau for higher concentrations and the readings at these levels are liable to be erroneous.

At first much trouble was encountered with the standardizing procedure of the microbiological assays (7, 8). It is known that the test organism used is particularly sensitive to soaps and detergents (9) as well as to growth factors which may either adhere to the glassware or be found in the distilled water or in some of the ingredients used to make up the medium. The first factor was checked by washing the test tubes, first with any type of soap, then with trisodium phosphate, then rinsing

[1] The culture was obtained from the Division of Nutrition, Food and Drug Administration, Washington, D. C.

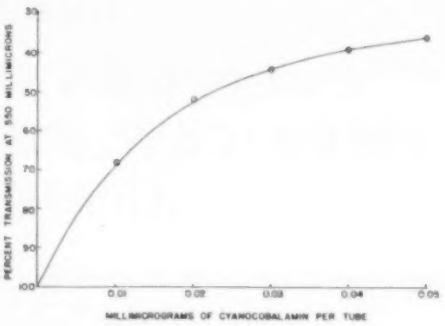


Fig. 1.—Standard curve.

abundantly with hot tap water and then rinsing again a minimum of seven times with distilled water. Prior to their use, the test tubes as well as the other glassware used for the preparation of the solution of media were sterilized for two hours in an oven at 175°.

On various occasions, upon completion of exposures, the solutions would respond to the microbiological assay by registering an apparent increase in B_{12} potency. This fact was particularly true when the solutions were exposed to direct sunlight where no protection whatever was given the cuvettes. The spectrophotometric method, on the contrary, held consistent results and in the case of sunlight treatment in particular, would always show a decrease of the vitamin concentration. At first, it was considered that the molecule of cyanocobalamin might be split by irradiation and that the broken-down products might prove more assimilable by the test bacteria. This surmise was, however, abandoned after replicate experiments showed a rate of destruction similar to that observed with the spectrophotometric method. Obviously, in the course of some experiments, the exposed solutions gathered airborne factors that enhanced the growth properties of the test organism. When encountered, such aberrant results were discarded on that basis.

Lighting Experiments

Exposure to Natural Light.—The following experiments include three intensities which are summarized in Table I.

TABLE I

Nature of Light	Light Intensity in Foot-Candles*
Direct sunlight	8,000 to 10,000
Daylight in shade	300
Dim daylight	10

* Foot-candles as read on a Weston Master II Model 735 Universal Exposure meter. For brightness above 1600 f.c. Kodak Wratten Filters of various densities were interposed.

Direct sunlight (Fig. 2).—All exposures were made between 11 a.m. and 1 p.m. on clear days when the brightness of the sunlight was practically constant. After irradiation, aliquots of the same solutions were analyzed, both by the bioassay and the spectrophotometric methods.

(*a*) *Bioassay curve.*—All points on this curve are the arithmetic mean of four separate experiments

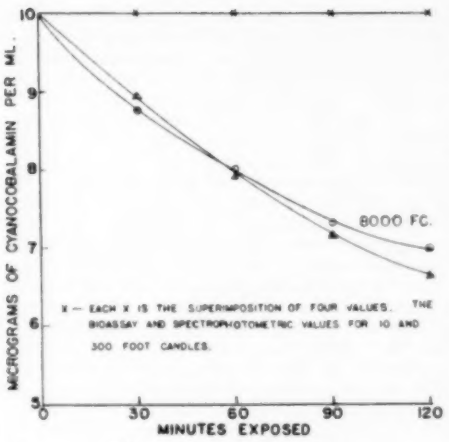

MICROGRAMS OF CYANOCOBALAMIN PER ML.
MINUTES EXPOSED
8000 FC.
X — EACH X IS THE SUPERIMPOSITION OF FOUR VALUES. THE BIOASSAY AND SPECTROPHOTOMETRIC VALUES FOR 10 AND 300 FOOT CANDLES.

Fig. 2.—Natural daylight.

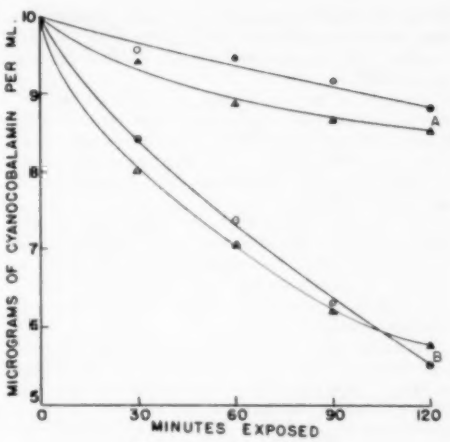


Fig. 3.—Artificial light.

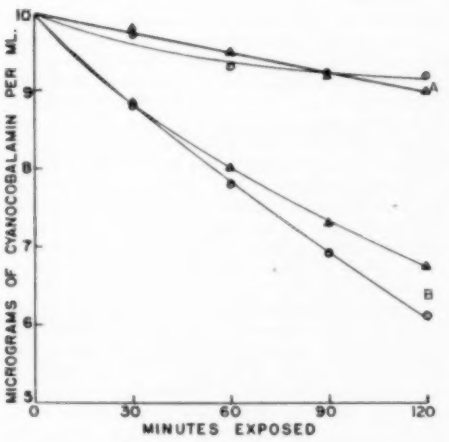


Fig. 4.—Signal purple.

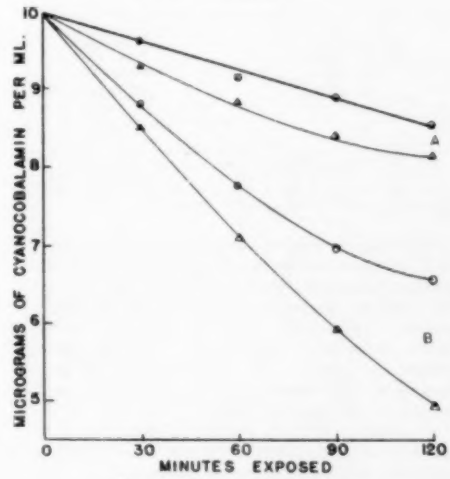


Fig. 5.—Light theater blue.

Legend: *A*—25 cm. from light source; *B*—12.5 cm. from light source; △—spectrophotometric assay; ⊙—bioassay.

and the values for each experiment are the mean of determinations at three different dilutions.[3] The standard deviation is, consequently, obtained in each case from a total of 12 figures (Table II).

(*b*) *Spectrophotometric curve.*—The results are essentially in agreement with the bioassay results. Each point on the spectrophotometric curve is the mean of four analyses of samples of the solutions used to establish the bioassay curve[3]. The standard deviations are, consequently, based on a total of four figures for each point (Table II).

[3] All subsequent bioassay curves were established in accordance with this procedure.

[3] All subsequent spectrophotometric curves were established in accordance with this procedure.

TABLE II

Bioassay		Spectrophotometric Assay	
Mean Value μg./ml.	S. D.	Mean Value μg./ml.	S. D.
8.7	1.40	8.9	0.19
8.0	0.41	7.9	0.17
7.4	0.62	7.1	0.15
7.0	0.30	6.7	0.23

Exposed to bright sunlight, neutral aqueous solutions of crystalline vitamin B_{12} lose approximately 10% of their potency for every half hour of direct exposure.

In addition to the quadruplicate experiments shown in Fig. 2, performed at a brightness between

8,000 and 8,200 foot-candles, single experiments were made at brightnesses of 10,000 and 12,000 foot-candles. The results showed slightly faster rates of destruction.

Daylight in the shade (Fig. 2).—A brightness of 300 foot-candles was easily maintained in the shade between the hours of 2:30 and 4:30 in the afternoon. The microbiological and spectrophotometric methods were in perfect agreement, and showed no destruction whatever.

Dim daylight (Fig. 2).—The brightness of the light was easily adjusted to 10 foot-candles in the laboratory. After two hours, no destruction of vitamin B_{12} had occurred and the potency curves are entirely superimposed on the ones obtained at 300 foot-candles.

Artificial light (Fig. 3).—A 300-watt reflector type filament lamp was used with an average voltage of 110. Variations of the light intensities were obtained by changing the distance between the light source and the surface of the solution (Table III).

TABLE III[a]

Distance Between Light Source and Solutions in cm.	Light Intensity in Foot-Candles
12.5	14,000
25	7,000
50	3,600

[a] A 5% transmittancy Kodak Wratten interference filter was used to obtain these values. The intensity does not follow the inverse square law mostly because it is a combination of point source and reflected light.

The bioassay curve of destruction for an intensity of 14,000 foot-candles is in good agreement with the spectrophotometric curve. The former shows a 45% loss of vitamin B_{12} potency after two hours of exposure against 42.5% where the spectrophotometric curve is concerned. For half the light intensity the loss is about 12% after two hours according to the bioassay, against 15% in the case of the spectrophotometric assay.

No destruction was observed after two hours' exposure at a distance of 50 cm. for a brightness corresponding to 3,600 foot-candles.

Quasi-Monochromatic Light Sources.—The same 300-watt reflector lamp was used in this series of experiments; Corning glass color filters were interposed between the solution and the light source. Preliminary experiments showed that studies at two distances, 12.5 cm. and 25 cm. between the surface of the solution and the light source, were sufficient. Irradiation at greater distances in all cases, would produce no significant destruction of B_{12}.

The brightness of these monochromatic light sources cannot be represented by absolute values. In order to state with accuracy the way the experiments were performed, and permit reproducibility, Table IV was composed. It gives the maximum transmission wavelengths of the filters. The Weston exposure meter was used to measure the brightness of the artificial light, first in the absence of any filter (Table III), then in conjunction with each one separately. The relative response or response to eye sensitivity (Col. 4, Table IV) was translated into the normalized responses.

Exposure to Signal Purple light, 400 mμ (Fig. 4).—The bioassay and spectrophotometric methods are in fair agreement for irradiations at 12.5 cm. and 25 cm., respectively. The average of values obtained for both assays after two hours shows a destruction of some 26% for a distance of 12.5 cm. and of slightly less than 9% at 25 cm.

Exposure to Theater Blue Light, 450 mμ (Fig. 5).—The trend of destruction is the same at 12.5 cm. and 25 cm. whether one considers the bioassay curves or the spectrophotometric results. In absolute values, the microbiological assays do not show as great a destruction rate. According to this method, the destruction amounts to 35% at 12.5 cm. after two hours and drops to 15% at 25 cm. The corresponding spectrophotometric values are 50% and 19%.

Exposure to Sextant Green light, 525 mμ (Fig. 6).—For both distances the bioassay and spectrophotometric methods are in close agreement. The average destruction after two hours' exposure amounts to less than 20% for a distance of 12.5 cm. and is negligible at 25 cm.

Exposure to Traffic-Shade Yellow light, 560 mμ (Fig. 7).—For both distances the two methods give similar results. As in the case of the exposure to green light, the bioassays show higher results than the spectrophotometric method at the same levels. It was expected that the destruction properties would decrease with an increase in wavelength. This view may still hold true, especially if one considers that the normalized response for yellow light is ten times that obtained for green light. It may safely be surmised that for equal brightness there would be

TABLE IV

Description and No. of Corning Color Filters	Characteristics of Colored Filters, Maximal Transmission, mμ	Distance from Light Source to Surface of Solution in cm.	Brightness in Foot-Candles, Relative Response[a]	Sensitivity Scale of Light Meter[b]	Normalized Response
Signal purple	400	12.5	1,200	0.52	1,720
#5551		25	900	0.52	1,710
Light theatre	450	12.5	1,550	0.76	2,040
blue #5031		25	1,250	0.76	1,645
Sextant green	525	12.5	700	0.98	715
#4010		25	550	0.98	560
Traffic shade	560	12.5	7,000	0.99	7,070
yellow #3484		25	5,400	0.99	5,455
Lantern red	610	12.5	3,000	0.85	3,530
#2412		25	2,500	0.85	2,940

[a] Foot-candles as read on a Weston Master II Model 735 Universal Exposure Meter.
[b] The relative sensitivity scale is obtained from the Weston Electrical Instrument Corporation for this type of cell normalized to unity at 550 mμ.

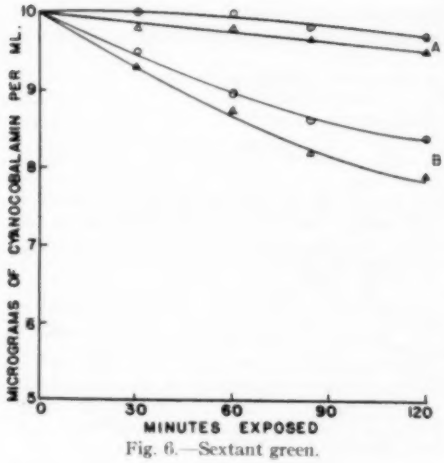


Fig. 6.—Sextant green.

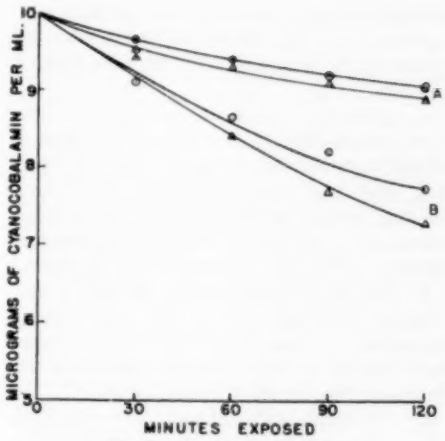
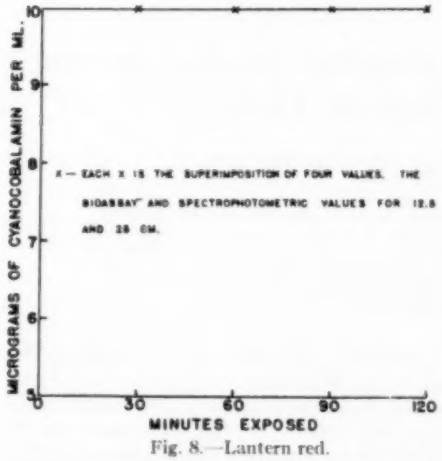


Fig. 7.—Traffic shade yellow.


MICROGRAMS OF CYANOCOBALAMIN PER ML.
MINUTES EXPOSED
X — EACH X IS THE SUPERIMPOSITION OF FOUR VALUES. THE
BIOASSAY AND SPECTROPHOTOMETRIC VALUES FOR 12.5
AND 25 CM.
Fig. 8.—Lantern red.

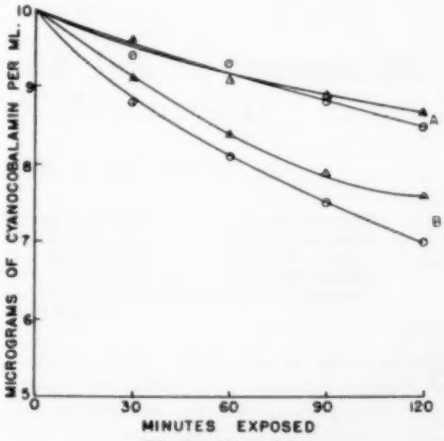


Fig. 9.—Ultraviolet.

Legend: *A*—25 cm. from light source; *B*—12.5 cm. from light source; △—spectrophotometric assay; ⊙—bioassay.

no detectable destruction of the vitamin. Under the present conditions, the destruction averages 25 % at 12.5 cm. and 10% at 25 cm.

Exposure to Lantern Red light, 610 mμ (Fig. 8).—There is no detectable loss of vitamin B_{12} at the two distances used. The microbiological and spectrophotometric methods are in perfect agreement.

Exposure to Ultraviolet Light (Fig. 9).—A low-voltage Mediquartz ultraviolet lamp[4] having the following characteristics was used:[5]

Spectral distribution:

2,000 to 2,600 A.U.	90%
2,600 to 3,000 A.U.	8%
3,000 to 3,200 A.U.	2%

Intensity in microwatts per square centimeter at 24-inch distance: 800.

The lamp was new when the present study started. It may consequently be assumed that no loss of efficiency occurred during the few hours it was put into service.

Although no factor of proportion could be established with the other results, the curves clearly show that the rate of destruction augments as the distance between the solution of B_{12} and the source of ultraviolet light decreases. The microbiological assays and the spectrophotometric results are in good agreement. Under the conditions described, the average destruction amounted to 14% at a distance of 25 cm. and to 28% at a distance of 12.5 cm.

For the conditions used in this study, the following conclusions may be drawn.

CONCLUSIONS AND SUMMARY

1. A sunlight intensity of approximately 8,000 foot-candles causes a 34% loss of potency

[4] Medical Quartz Products Co., Los Angeles, Calif.
[5] Information furnished by the manufacturer.

in neutral solutions of vitamin B_{12} after two hours' exposure. For and below 300 foot-candles no destruction is noticeable after the same time of exposure.

2. Light from an incandescent reflector lamp of approximately 14,000 foot-candles has a marked destructive effect on B_{12} solutions (some 45% after two hours' exposure). This value decreases rapidly as the intensity of the light decreases. A brightness of 3,600 foot-candles causes no detectable alteration after two hours.

3. For monochromatic light sources, whose corrected intensities are in the same range, destruction is more efficient in the short wavelength region. A high light intensity in the red showed no destruction after two hours.

4. The results obtained with ultraviolet light follow the pattern obtained for sunlight. A quantitative relationship is not presented since comparative brightness cannot be established.

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Pharmaceutical and Toxicological Studies on the Polyuronide of Sapote Gum*

By WOODROW R. BYRUM† and L. B. DALE, Jr.‡

Pharmaceutical and toxicological studies were made of the polyuronide obtained from sapote gum. The gum proved to be of value as an emulsifying and suspending agent. No toxicity was demonstrated in rabbits, mice, and rats after its intraperitoneal, intravenous, and oral administration in high-level single doses. It proved not to give rise to irritation when tested on skin and mucous membranes in rabbits, guinea pigs, and humans.

SAPOTE GUM[1] is an amber colored, brittle, resinous substance with a lustrous appearance. The gum is obtained by incising the trunk of *Achras sapota* (*Sapotaceae*) a tall evergreen tree cultivated in tropical America as a source of chicle. When the trunk of the tree is tapped, the latex which is exported as crude chicle, flows for a time. As the flow of latex ceases, sapote gum slowly forms over the wound. After hardening, the gum is cut from the tree with machetes and shipped in commerce containing varying amounts of impurities. There is an abundant and inexpensive supply of the sapote gum in Peru, and the yield has been estimated to be from 100 to 200 tons a year (1). Sapote gum is used in Peru as a sizing material in the manufacture of textiles and felt hats, as a glue, and for the preparation of water paints.

Anderson and Ledbetter (2) reported that sapote gum is a mixture of 90 to 94 per cent water-soluble polyuronide and 6 to 10 per cent water-insoluble resin. The two substances can be separated by dissolving the polyuronide in hot water and filtering off the resin.

Since sapote gum has been found to possess emulsifying and suspending properties, it was deemed of importance to compare these properties with those of other agents commonly used for the same purpose and to determine if it possesses toxic or irritant properties.

The polyuronide of the gum obtained by the method of Anderson and Ledbetter (2) was used in all these studies and is referred to as purified sapote gum.

EXPERIMENTAL

Emulsifying Properties.—Preliminary studies indicated that the purified gum yielded more pharmaceutically acceptable and stable products than those produced by the crude gum. These preliminary studies also revealed that emulsions of the purified gum could be easily prepared by the addition of

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[1] The gum investigated and the information concerning its collection and uses were supplied by Asher, Kates and Company, Lima, Peru.

water and trituration, whereas emulsions were prepared from the crude gum by this method with difficulty.

In order to determine the optimal concentration of purified sapote gum needed for preparing emulsions of the type used, several were prepared using varying amounts of the gum. These were centrifuged at 3,000 r. p. m. and checked at one-, two-, and five-minute intervals. From results of this test it would not be advisable to reduce the amount of gum below ten per cent for the preparation of emulsions. A total concentration of 1:500 methyl and propyl *p*-hydroxybenzoates was used as preservative in all emulsions. All emulsions were made using the dry gum method and prepared in a Waring blender. After preparation, the emulsions were homogenized in a hand homogenizer. The emulsions prepared from the purified sapote gum were creamy-white in appearance and practically odorless.

Emulsions of cottonseed oil and of liquid petrolatum in 30% concentrations were prepared using acacia, tragacanth, and purified sapote as the emulsifying agents in concentrations of 12, 1, and 12% respectively (Table I). Six 2-ounce samples were made of each formula and tested under the following conditions: (*a*) Shelf at room temperature, 23°. (*b*) Oven at 40°. (*c*) Refrigerator at 3°. Observations were made daily for three days and then at three-day intervals for twenty-eight days, and are recorded in Table I. The end point was taken as that day on which a noticeable separation of the emulsion had occurred.

To check further stability of the various emulsions prepared, each emulsion was centrifuged at 3,000 r. p. m. and checked at intervals of five minutes or until broken. Results are tabulated in Table I. Hollenberg (3) has shown that five minutes of centrifuging at 3,000 r. p. m. is equivalent to approximately one week's shelf life.

Suspending Properties.—The suspending properties of purified sapote gum were compared with those of bentonite magma and tragacanth. The agents suspended were zinc oxide, 10%, and kaolin, 10%. The emulsifying agents were used in the following concentrations: sapote gum, 10%; bentonite magma, 25%; and tragacanth, 1%. The same procedures, in general, were followed in the preparation of the suspensions as were used by de-Navarre and Koehler (4), DeKay and Osborn (5), and Pfeiffer and Williams (6). Three 2-oz. samples of each suspension were prepared and stored at room temperature (23°). Observations were made at the end of one-, seven-, and fourteen-day intervals. Each observation consisted of a macroscopic examination and a pour test. The macroscopic examination was an attempt to determine the presence of any settling or separation. One of the three samples was gently poured into a beaker during each observation so that any settling could be detected.

TABLE I.—STABILITY OF EMULSIONS UNDER VARIOUS CONDITIONS

Emulsion	—Storage Conditions— Shelf 23°C. Days	Oven 40°C. Days	Refrig. 3°C. Days	Centrifugation[a] Minutes
Liquid petrolatum 30% and tragacanth 1%	16	22	>28	5
Cottonseed oil 30% and tragacanth 1%	19	19	>28	5
Cottonseed oil 30% and acacia 12%	>28	>28	>28	15
Liquid petrolatum 30% and acacia 12%	22	16	>28	20
Liquid petrolatum 30% and purified sapote 12%	25	20	>28	30
Cottonseed oil 30% and purified sapote	>28	>28	>28	>35

[a] 3,000 r. p. m.

The results of these preliminary suspending tests indicated that sapote gum compared favorably with tragacanth and bentonite magma as a suspending agent. Additional studies are being pursued on this property of sapote gum in which microscopic examinations of the suspensions are being made. The information obtained warrants this more extensive study.

Influence of *p*H on Viscosity of Purified Sapote, Acacia, Tragacanth, and Pectin Solutions.—One per cent w/v solutions of purified sapote, acacia, tragacanth, and pectin were made in a Waring blender, agitating each for five minutes. A total concentration of 1:500 methyl and propyl *para*-hydroxybenzoates was used as preservative. The *p*H of each solution was determined by means of the Beckman *p*H meter, and the relative viscosity was ascertained by determining the time, in seconds, required for 50 cc. of the solution to flow through a standard opening (7). All work was carried out at a room temperature of 30 to 32°. The normal *p*H and viscosity for each solution were determined and are recorded in Table II. Five samples of each solution were adjusted to *p*H values of 3, 5, 7, 9, and 11, ± 0.1, using 1.0 *N* sodium hydroxide or 1.0 *N* hydrochloric acid solutions. Six determinations of the viscosity of each of the adjusted solutions were made. The average values are recorded in Table II. All solutions turned dark with an increase in *p*H with sapote solutions turning the darkest. Sapote, acacia, and tragacanth developed a formaldehyde-like odor at *p*H of 7, 9, and 11 which was most pronounced in the sapote solutions.

Acute Toxicity in Mice.—The acute intravenous and intraperitoneal toxicities of a freshly prepared 10% solution of purified sapote gum were determined in albino mice. Male, Swiss strain, albino mice weighing 18 ± 2 Gm. were used in this test. Ten animals were used per dosage level with a minimum of three levels per determination. The animals were administered 1.0, 1.5, and 2.0 Gm./Kg. intravenously and 1.5, 2.0, and 3.0 Gm./Kg. intraperitoneally. Higher concentrations were not attempted due to the viscosity of the resulting mucilage. Observations were made at 3, 12, 36, and 72 hours. No deaths occurred and no toxic reactions were noted after the administration of the gum by either route.

Acute Toxicity in Rats.—The acute oral toxicity of purified sapote gum, after single administration, was determined in male, albino rats weighing 100 ± 20 Gm. The compound was administered in the form of a freshly prepared 10% solution by means of a stomach tube which consisted of a French No. 8 catheter connected to a 5-cc. syringe. Three

TABLE II.—THE INFLUENCE OF pH ON VISCOSITY[a] OF ACACIA, PURIFIED SAPOTE, TRAGACANTH, AND PECTIN SOLUTIONS

Solutions 1% w/v	Normal pH	Normal Viscosity, Seconds	Viscosity of Adjusted Solutions, Seconds				
			pH 3	pH 5	pH 7	pH 9	pH 11
Acacia	5.2	63.0	59.7	62.9	60.1	61.1	61.0
Sapote	5.0	64.3	62.5	64.8	64.2	64.5	63.4
Tragacanth	6.0	376.3	390.8	477.0	972.0	190.5	157.2
Pectin	3.5	153.6	152.2	155.2	128.6	144.0	79.8

[a] Viscosity is expressed as the number of seconds required for 50 cc. of solution to flow through a standard opening. Water required 58.4 seconds under the same conditions.

groups of 10 rats each were given 2.0, 3.0, and 5.0 Gm./Kg. Observations were made at 3, 12, 36, and 72 hours, and then daily for fourteen days. No deaths occurred and no toxic reactions were noted after the administration of the gum.

The acute toxicity of purified sapote gum, after single intraperitoneal administration, was investigated in albino rats weighing 75 to 125 Gm. each. The compound was administered in the form of a freshly prepared 10% solution. Three groups of 10 rats each were given 1.0, 1.5, and 2 Gm./Kg. of body weight. The rats were under continuous observation during the first four hours following administration, and then several times daily for fourteen days. No toxic reactions were observed immediately after the injection or during the fourteen day observation period.

Acute Toxicity in Rabbits.—Purified sapote gum was administered in the form of a freshly prepared 10% solution to five rabbits by stomach tube each receiving 2.5 Gm./Kg. The rabbits were observed frequently during the first four hours following administration, and then daily for thirty days. The test was repeated on the same rabbits administering the same quantity of sapote gum at the end of the thirty-day observation period. At no time were ill effects observed.

Topical Irritation and Toxicity.—Sapote mucilage (15%) was investigated in order to determine if deleterious effects would occur when applied topically in rabbits, guinea pigs, and humans.

Animal Mucous Membrane.—Application to the penile mucosa and eye of the rabbit was carried out as described by Draize, *et al.* (8). Approximately 0.2 cc. of the 15% mucilage of purified sapote gum was required for application and thorough wetting of the penile mucosa. Examination was made at 2, 12, and 24 hours after application. No evidence of irritation (erythema and edema) was noted.

In the measure of injury to the eye, 0.1 cc. of the mucilage was instilled in the conjunctival sac. Observations were made at 1, 24, and 48 hours after instillation. No irritation to the corneal or conjunctival membranes occurred. Ten animals were used in each of the above tests.

Human Skin.—The use of the patch test in the evaluation of primary irritation and of contact dermatitis as reviewed by Schwartz and Peck (9) and Draize, *et al.* (8), was carried out. Forty-five students, both male and female, were used in this test. One-tenth gram of 15% purified sapote gum in white petrolatum was applied to patches placed on the medial surface of the upper left arm and allowed to remain for 24 hours. One-tenth gram of 15% pectin in white petrolatum was applied in a similar manner to the right arm as an arbitrary reference. No irritation as determined by the absence of erythema or edema was noted from either the sapote gum or the reference.

Sensitization.—The technique used was essentially that of Draize, *et al.* (8). The hair was removed by means of a fine-bladed clipper from the backs of 10 albino male guinea pigs weighing between 150 and 200 Gm. The first injection consisted of 0.05 cc. of a 0.1% freshly prepared solution of purified sapote gum given intracutaneously using a 26-gage needle. The remaining injections consisted of 0.1 cc. of 0.1% solution and were made three times weekly until a total of 10 injections had been given. Two weeks after the last injection a test injection was made using 0.05 cc. of a 0.1% solution. Readings of the test injections were made after 24 hours. Both the first injection and the test injection produced well defined erythema, but no edema occurred in any of the animals. The diameter and color of the reactions of the first and test injections were of approximately the same order. It was concluded, therefore, that no sensitization had taken place.

SUMMARY

Pharmaceutical and toxicological studies were made of the polyuronide obtained from sapote gum. The gum proved to be of value as an emulsifying and suspending agent. No toxicity was demonstrated in rabbits, mice, and rats after its intraperitoneal, intravenous, and oral administration in high-level single doses. It proved not to give rise to irritation when tested on skin and mucous membranes in rabbits, guinea pigs, and humans.

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A Comparison of Dichloralphenazone and Chloral Hydrate*

By W. B. RICE and J. D. McCOLL

Dichloralphenazone, a molecular complex of chloral hydrate and phenazone, has been compared with chloral hydrate on the basis of acute and chronic toxicity, the Hypnotic Dose 50, the onset and duration of hypnosis in rats, mice, and guinea pigs. The relative analgetic and antipyretic actions of these two compounds have been tested in mice and in rabbits respectively. Dichloralphenazone possesses approximately the same hypnotic properties as chloral hydrate and has desirable physical characteristics for pharmaceutical practice.

DICHLORALPHENAZONE, a molecular complex of two moles of chloral hydrate and one mole of phenazone, has recently been employed clinically as a sedative. Several combinations of chloral hydrate and phenazone were described as early as 1892 by Behal and Choay (1) and were known in the older literature as "hypnal" and "bihypnal."

These compounds have been investigated mainly for their antipyretic (2, 3, 4) and hypnotic actions by several workers. Steinmetzer (5) first reported the synergistic sedative effect between chloral hydrate and phenazone. These compounds never came into widespread therapeutic use, despite the fact that dichloralphenazone possesses physical properties which eliminate the unpleasant odor, disagreeable taste, and difficulty of pharmaceutical compounding generally associated with chloral hydrate.

The introduction of barbiturates was undoubtedly one reason why dichloralphenazone was neglected. The recent revival of chloral hydrate as a sedative has, in turn, initiated a re-evaluation of dichloralphenazone (6). The present investigation was undertaken prior to these clinical trials to compare the relative toxicity of chloral hydrate and dichloral phenazone as well as the hypnotic, analgetic, and antipyretic actions.

EXPERIMENTAL[1]

Acute Toxicity.—The acute intraperitoneal and oral[2] toxicity was determined in the female (strain CF-70) mouse (18–22 Gm.) for chloral hydrate, dichloralphenazone, and phenazone. Acute oral[2] toxicity was also estimated in the female (Sprague-Dawley) rat (180–200 Gm.). The number of animals which died at each dose level was recorded. From these data, expressed as percentage mortality, the LD_{50} in terms of mg./Kg. was calculated by the probit method (7). Although all deaths occurred within 48 hours, the surviving animals were kept under observation for ten days.

Chronic Toxicity.—The chronic toxicity was determined by the repeated administration of a hypnotic dose of chloral hydrate and dichloralphenazone twice weekly, to the same group of female (Sprague-Dawley) rats (average weight 200 Gm.) for a period of eight weeks. The drugs were administered by the oral route in a 4% solution following an 18-hour fasting period. The dosage schedule was as follows:

	(2 weeks)	(6 weeks)
Chloral Hydrate	300 mg./Kg.	400 mg./Kg.
Dichloralphenazone	450 mg./Kg.	600 mg./Kg.
Phenazone	150 mg./Kg.	200 mg./Kg.
Control	H_2O	H_2O

The body weight of each animal was recorded daily during the experimental period. A blood smear was taken from the tail of each animal before treatments commenced and at the conclusion of the experiment.

All animals were sacrificed and examined for gross pathological changes. A tibial bone marrow smear was taken from each animal at the time. A differential blood count was done on each sample of blood and bone marrow by the usual methods.

Hypnotic Action.—The HD_{50} was estimated in female mice (18–22 Gm.) and in male and female rats (180–200 Gm.) following the intraperitoneal administration of these compounds. A Latin Square design was employed to control the order of the treatments and the percentage of animals which lost their righting reflex at each dose level was recorded. These quantal response data were then used to calculate the HD_{50} by the probit method (7).

The onset and the duration of hypnosis was recorded and these data compared by analysis of variance. The duration of hypnosis was taken as the time in minutes during which the righting reflex was abolished. Similarly, the onset of hypnosis was the time required to produce the loss of this reflex.

The female guinea pig (200–250 Gm.) was also used to study the onset and the duration of hypnotic action. The drugs were compared following intraperitoneal administration at two dose levels each.

A Latin Square design was chosen to compare the effects of several doses of phenazone administered separately on the duration of the hypnotic action of chloral hydrate. Four groups of four female rats

* Received March 28, 1955, from the Research Laboratories, Frank W. Horner Limited, Montreal, Canada.

"Chlorol" is The Registered Trademark of Frank W. Horner Ltd. for Dichloralphenazone.

[1] Mice and rats were maintained on Purina "Fox-Chow Checkers" and water; rabbits and guinea pigs on Ogilvie "Miracle" rabbit food and water supplemented with fresh carrots and cabbage. Room temperature was maintained at 21° for all experiments which were commenced in the forenoon.

[2] Mice and rats were deprived of food for twelve hours before oral medications, unless otherwise specified.

TABLE I.—COMPARISON OF THE ACUTE AND CHRONIC TOXICITY FOR CHLORAL HYDRATE, DICHLORALPHENAZONE AND PHENAZONE IN THE RAT AND IN THE MOUSE

	Acute Toxicity					Chronic Toxicity			
	Species	Route	Number	LD_{50} mg./Kg.	95% Fiducial Limits	Route	Number	Weight Gain Gm. ± S.D.[a]	P[b]
Control	Rat	...	..	...	...	oral	7	22.2 ± 10.0	..
Chloral hydrate	Mouse	i. p.	20	777	774–781				
Chloral hydrate	Mouse	oral	50	1116	1102–1131				
Chloral hydrate	Rat	oral	45	955	690–1320	oral	7	17.3 ± 9.9	0.9
Dichloralphenazone	Mouse	i. p.	40	982	910–1060				
Dichloralphenazone	Mouse	oral	50	1437	1331–1551				
Dichloralphenazone	Rat	oral	39	1338	1195–1497	oral	8	14.9 ± 5.8	0.10
Phenazone	Mouse	i. p.	36	1032	983–1084				
Phenazone	Mouse	oral	24	1310	1197–1434				
Phenazone	Rat	...	..	...	...	oral	8	24.5 ± 12.6	...

[a] Standard deviation = $\sqrt{\frac{\Sigma(X_i - \bar{X})^2}{N - 1}}$ (8).

[b] "T" test for small samples = $\frac{m_1 - m_2}{S\sqrt{1/N_1 + 1/N_2}}$ where $S = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}$ as compared with controls (8).

TABLE II.—DIFFERENTIAL BLOOD LEUKOCYTE COUNTS[a] OF FEMALE RATS BEFORE AND AFTER EIGHT-WEEK CHRONIC TOXICITY TEST

	Number Animals	Lymphocytes %	Monocytes %	Total Mono-Nuclears %	Neutrophils %	Eosinophils %	Basophils %	Total Granulocytes %
Before Treatment								
Chloral hydrate	8	77.1	6.9	84.0	12.3	3.0	0.9	16.2
Dichloralphenazone	8	80.1	3.7	83.8	12.8	3.2	0.3	16.3
Phenazone	8	81.9	2.3	84.2	13.6	2.2	0.7	16.5
After Treatment								
Chloral hydrate	7	82.2	2.7	84.9	12.6	2.9	0	15.5
Dichloralphenazone	8	71.4	3.8	75.2	14.7	4.5	0	19.2
Phenazone	8	78.6	3.9	82.5	13.2	4.2	0	17.4

[a] Figures are means of two differential counts on each animal.

(180–200 Gm.) each were employed. All groups received, by the intraperitoneal route, 300 mg./Kg., of chloral hydrate. Doses of phenazone (100, 200, and 300 mg./Kg.) were randomly administered to three groups; the fourth group served as control. The data were examined by analysis of variance.

Analgetic Action.—The analgetic effect produced by chloral hydrate and the stoichiometric equivalent dose of dichloralphenazone was determined in the female mouse (18–22 Gm.) using the method described by Burn (7). This involves the stimulation of the mouse tail by an interrupted electric current. A Latin Square design was employed and the percentage of animals which did not respond to ten or more "shocks" was recorded.

Antipyretic Action.—Hyperthermia was produced in the female rabbit (2.8–3 Kg.) by the intravenous injection of autoclaved *Escherichia coli* suspension.[3] The Randomized Block design was employed. The suspension was injected (1 ml./Kg.) and one hour later, when hyperthermia was produced, the compounds were administered by the intraperitoneal route. Rectal temperatures were recorded at hourly intervals.

RESULTS

Toxicity.—Table I summarizes the acute toxicity data for chloral hydrate, phenazone and dichloralphenazone by the oral and intraperitoneal route. Chloral hydrate was more toxic than dichloralphenazone by both routes on a weight basis. The toxicity of dichloralphenazone was proportional to the molecular content of chloral hydrate. The two compounds demonstrated the same relative toxicity between species. They appear to be absorbed from the gastrointestinal tract to the same extent on the basis of the small difference between the intraperitoneal and oral LD_{50}.

The change in body weight during the period of the eight-week chronic toxicity test is also shown in Table I. All groups continued to gain weight for the duration of the test. These data based on the increased body weight over initial weight of each group were compared by the "t" test (8). There was no significant difference between the treated groups and the control group at the 5% level, although the dichloralphenazone group approached a significant level ($P = 0.10$). However, there was no difference between the weight gain of the dichloralphenazone group and that of the chloral hydrate group.

Table II shows the results of the differential blood leukocyte count before and after the treatment periods in the chronic test. No essential difference was observed except for the complete absence of basophils in all groups after the treatment period. There was no evidence of granulocytic depression. Examination of the tibial bone marrow

[3] Kindly supplied by Mr. E. Isenberg.

TABLE III.—COMPARISON OF HYPNOTIC ACTION[a] OF CHLORAL HYDRATE AND DICHLORALPHENAZONE FOLLOWING INTRAPERITONEAL ADMINISTRATION

	Species	Number	Dose mg./Kg.	HD_{50} mg./Kg. (95% Fiducial Limits)	Number Responding	Onset of Hypnosis[b] (Min. ± S.D.)	Duration of Hypnosis[b] (Min. ± S.D.)	Therapeutic Index (LD_{50}/HD_{50})
Chloral hydrate	Mouse	20	...	282(272–292)	10	6.4 ± 1.0	23.0 ± 10.8	2.7
	Guinea Pig	48	250	...	48	3.5 ± 2.7	73.0 ± 11.7	...
	Guinea Pig	48	350	...	48	3.6 ± 2.7	96.0 ± 11.7	...
	Rat	15	200	218(211–226)	5	4.4 ± 1.4	21.9 ± 18.7	...
	Rat	35	300	...	29	4.3 ± 1.6	51.0 ± 9.0	...
	Rat	59	400	...	59	5.8 ± 3.1	144.0 ± 13.2	...
Dichloralphenazone	Mouse	20	...	450(445–456)	50	7.9 ± 1.1	16.0 ± 10.0	2.2
	Guinea Pig	48	375	...	32	3.4 ± 2.7	52.0 ± 11.7	...
	Guinea Pig	48	525	...	40	3.8 ± 2.7	88.0 ± 11.7	...
	Rat	14	300	435(408–463)	2	5.6 ± 1.1	27.0 ± 16.0	...
	Rat	35	450	...	22	5.7 ± 5.3	68.0 ± 8.7	...
	Rat	57	600	...	57	4.9 ± 1.6	205.0 ± 9.2	...

[a] Loss of righting reflex. [b] Mean response of total group tested at each dose level.

smear demonstrated good granulocytopoietic activity in all groups.

There were no gross pathological differences observed in the heart, liver, kidney, spleen, or stomach of any of these animals.

Hypnotic Action.—The HD_{50}, the onset and the duration of hypnosis in the mouse following intraperitoneal administration of the drugs are shown in Table III. The HD_{50} for chloral hydrate was estimated to be 282 mg./Kg. while that for dichloralphenazone was 450 mg./Kg. Based on the molecular content of chloral hydrate, the "expected HD_{50}" would be 405 mg./Kg. for dichloralphenazone. This "expected HD_{50}" was found significantly different from the observed value for this compound indicating that slightly more of dichloralphenazone was required than of chloral hydrate to produce a given response in the mouse. The time of onset of hypnosis appeared to be longer with dichloralphenazone but this difference was not significant. It was also suggested that this drug had a shorter period of hypnotic action but again this difference was not statistically significant as compared to the response with chloral hydrate.

The therapeutic indexes calculated from the ratio of the LD_{50}/HD_{50} were of the same order and further demonstrated that these compounds are very similar in their action in this species.

In the guinea pig, comparison of the hypnotic action (Table III) at two dose levels demonstrated that the onset of hypnosis was more rapid, while the duration of action was longer than in the mouse. There was no significant difference between the period of onset at any dose level for either compound. The duration of hypnosis was significantly longer at the low dose of chloral hydrate compared with the stoichiometric equivalent dose of dichloralphenazone. There was no significant difference between the responses of these compounds at the higher dose levels.

In the rat, the HD_{50} for these compounds was found to be of the same relative order as in the mouse. Again the HD_{50} for dichloralphenazone was slightly larger than expected on the basis of the molecular content of chloral hydrate. The onset of hypnosis in the rat appeared to be the same with both drugs and no statistical difference was observed. The duration of hypnotic action, however, was found to be significantly greater with dichloralphenazone compared with the stoichiometric equivalent doses of chloral hydrate. This prolongation of hypnosis was statistically significant at the higher dose levels and suggests an essential difference in the response of the rat to dichloralphenazone compared to the mouse or the guinea pig.

The effect of phenazone on the hypnotic action of chloral hydrate when administered separately by the intraperitoneal route in the rat is shown in Table IV. Chloral hydrate at a dose level of 300 mg./Kg. resulted in the mean loss of righting reflex of 82 minutes. The simultaneous injection of phenazone at doses of 100, 200, and 300 mg./Kg. significantly prolonged the duration of this hypnotic action. No significant difference in duration was observed by increasing the dose of phenazone from 100 to 200 mg./Kg.; an increase in duration was observed with the 300 mg./Kg. dose as compared with the response from 100 mg./Kg.

Analgetic and Antipyretic Actions.—Table V summarizes the results of the comparison of the analgetic and antipyretic action of these compounds. Analysis of the data obtained demonstrated no significant difference between the analgetic response of the control animals and those receiving chloral hydrate

TABLE IV.—EFFECT OF PHENAZONE ON DURATION OF HYPNOSIS[a] PRODUCED BY CHLORAL HYDRATE IN RATS

Chloral Hydrate (i. p.)	Phenazone (i. p.)	Duration of Hypnosis (Min.)	95% Necessary Difference[b] (Min.)
300 mg./Kg.	...	82	18
300 mg./Kg.	100 mg./Kg.	111	18
300 mg./Kg.	200 mg./Kg.	117	18
300 mg./Kg.	300 mg./Kg.	131	18

[a] Loss of righting reflex. [b] Difference by which values must vary to be significant from controls (8).

TABLE V.—ANTIPYRETIC RESPONSE IN FEMALE RABBITS AND ANALGETIC RESPONSE IN FEMALE MICE

	Dose (mg./Kg.) i. p.	Antipyretic[a] Response	Analgetic Response (% Animals)[b]
Control	...	+3.0°	6.8
Chloral hydrate	250	...	6.8
Chloral hydrate	300	−1.5°	...
Chloral hydrate	350	...	10.0
Dichloralphenazone	400	−1.8°	...
Dichloralphenazone	450	...	20.0
Dichloralphenazone	550	...	53.4
Phenazone	100	−1.1°	...

[a] Mean response for 3 hours of 12 determinations.
[b] Per cent of mice not responding to 10 or more "shocks."

at doses of 250 or 350 mg./Kg. The response of the groups receiving dichloralphenazone, compared with the control group, demonstrated no essential difference at the 450 mg./Kg. dose. However, the response at the 550 mg./Kg. dose was highly significant at the 1% level. This analgetic effect presumably is due to the phenazone content of the dichloralphenazone, although the former compound was not tested separately in this experiment.

All treatments were effective in reducing the hyperthermia produced by the administration of *E. coli* suspension over a three-hour period. The control animals demonstrated a rise in temperature of 2–3°F. The antipyretic effect observed with dichloralphenazone (400 mg./Kg.) was greater than that with chloral hydrate (300 mg./Kg.) or with phenazone (100 mg./Kg.). The degree of hypothermia produced by dichloralphenazone under these conditions was not essentially different from that expected by the additive effect produced with chloral hydrate and phenazone alone.

DISCUSSION AND CONCLUSIONS

The acute toxicity of dichloralphenazone was less in rats and mice than was chloral hydrate on a weight basis and approximately proportional to the content of chloral hydrate. The values for the LD_{50} of chloral hydrate are in good agreement with those reported by Adams (9).

The chronic toxicity test in the rat did not suggest that treatment with dichloralphenazone, phenazone, or chloral hydrate resulted in any observable toxic manifestations under the conditions of this test. These data are in accord with those of Adams (9) for chloral hydrate. Examination of the literature reveals that reports of secondary agranulocytosis resulting from phenazone are rare. Although aminopyrine has been indicated as a causative agent, Greenberg (10) points out that the methylamino linkage, which appears to play an important part in the agranulocytic action of pyrazolone derivatives, is lacking in phenazone.

The comparison of dichloralphenazone and chloral hydrate by the hypnotic dose, by the onset and the duration of hypnosis and by the therapeutic indexes in the mouse, guinea pig, and rat indicates that these compounds possess similar hypnotic properties. There was some evidence in the mouse and in the guinea pig that the action of dichloralphenazone was of shorter duration. In the rat, however, the duration of action was found to be longer than that of stoichiometric equivalent dose of chloral hydrate.

Chloral hydrate acts as a hypnotic largely because it is rapidly transformed to trichloroethanol in the body (11). It is this latter metabolite which is hypnotic. The phenazone component of dichloralphenazone is likely released before the conversion of chloral hydrate occurs. It is possible that such a preliminary transformation in the metabolism of dichloralphenazone could account for the HD_{50} being greater than that expected on the basis of the chloral hydrate content.

The prolongation of the hypnotic action of chloral hydrate by the administration of phenazone has been described (12, 3, 5). It is of interest to note that this action occurs in the rat whether the compounds are administered separately by the same route or as the molecular complex, dichloralphenazone. This may be the result of competition of trichloroethanol and phenazone for the same detoxification pathway in the body.

There appears to be some variation between species to the hypnotic response with chloral hydrate and dichloralphenazone. It has been generally accepted that the rat is the most suitable animal for hypnotic studies (13). These effects noted may be due to a different rate of metabolism in the mouse and in the rat. Marshall and Owens (11) have recently pointed out a marked variation in the metabolism of trichloroethanol in dog and in man. The existence of such dissimilarities between the mouse and the rat and their effect upon the duration of hypnosis is problematical.

The potentiation of the antipyretic action of chloral hydrate and phenazone, as reported by previous workers (2, 3), was not observed in this study with dichloralphenazone. This may be due to the different conditions of the test employed. The drugs were compared in this investigation for their capacity to reduce a labile fever. Previous workers had tested them on the basis of preventing the occurrence of a hyperthermia. The antipyretic action of dichloralphenazone was no greater than was observed with similar doses of chloral hydrate and phenazone under these conditions.

SUMMARY

Dichloralphenazone appears to possess all the hypnotic properties of chloral hydrate. It is comparable to chloral hydrate in terms of acute and chronic toxicity, antipyretic action, and, due to its content of phenazone possesses some mild analgetic properties. Because of its physical characteristics (i.e., a stable, crystalline solid) it may be easily administered in tablet form and is free of the odor and unpleasant taste associated with chloral hydrate.

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The Effect of Various Substances on the Antibacterial Activity of Bacitracin in Ointments*

By JAMES M. PLAXCO, Jr.,† and WILLIAM J. HUSA

The stability and relative availability of bacitracin alone and in mixtures in various ointment bases was determined. An attempt was made to correlate bacitracin potency as determined by a slightly modified F. D. A. method with the antiseptic efficacy of the ointments as measured by the agar plate diffusion method.

BACITRACIN ointment has been widely used the past few years in the treatment of numerous pyogenic skin infections. The recent trend has been to combine two or more antibiotics or other antiseptic agents in an ointment to increase the effectiveness of the ointment. Bacitracin has been mixed with penicillin, tyrothricin, polymyxin, and sulfacetamide. Reports of mixtures of bacitracin with the older antiseptics such as sulfathiazole, phenol, and mercury bichloride have not been found.

Hydrocarbon bases have been used generally but at least one other base, composed principally of diesters of polyethylene glycol 400 has been recommended for bacitracin and polymyxin ointment (1). Bacitracin is reasonably stable in anhydrous hydrocarbon bases but the drug is released rather slowly by the base. As reported previously (2), even though the amount of drug applied was sufficient, therapeutic failure resulted because release of the drug was too slow to permit bactericidal concentrations at the infected site.

The present F. D. A. method for the assay of bacitracin in ointments is a measure only of bacitracin potency and does not determine whether the drug is available or whether release and diffusion of the antibiotic through the agar is inhibited or prevented due to precipitation, adsorption, or some other factor.

The present investigation attempted to correlate bacitracin potency as determined by the F. D. A. method (with minor modifications) with the results obtained by the agar plate diffusion method for the determination of the antiseptic efficacy of ointments. The purpose of the investigation was to determine the stability and relative availability of bacitracin in various ointment bases, and to determine the effect of various substances on the antibacterial activity of bacitracin in ointments.

EXPERIMENTAL

Materials.—Bacitracin[1] having a potency of 50.0 u./mg. was used. Dehydrated Penassay seed and Penassay base agar were used as the media. The inoculum was prepared by washing the growth of a 24-hour culture of *Micrococcus pyogenes* var. *aureus* from a seed agar slant, standardizing the suspension in a photoelectric colorimeter, and adding 1 ml. of the standardized suspension to each 125 ml. of seed agar at 45°. Twenty milliliters of base agar was placed in each flat bottom Petri dish, allowed to harden, and layered with 4 ml. of inoculum.

Procedure

Determination of the bacitracin potency of the ointments involved extraction of the antibiotic from the base, and assay of the solutions so obtained by a method similar to that in the F. D. A. regulations (3). The extracted solutions were diluted to an estimated concentration of 2.5 u./ml. and placed on the seeded plates using Vincent disks. After incubating for 16–18 hours at 35°, the zones of inhibition were measured. Bacitracin potency was determined by comparing these zones with a standard curve obtained from standard bacitracin solutions. Extraction solutions from ointments containing less than 40% of theoretical potency at the time of assay produced no inhibition zones and the results were interpreted as zero potency.

Antiseptic efficacy of the ointments was determined by placing the ointments on the seeded plates by the use of Vincent disks, incubating, and measuring the zones of inhibition. Preliminary experi-

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[1] Contributed by S. B. Penick and Company.

TABLE I.—EFFECT OF VARIOUS OINTMENT BASES ON THE ACTIVITY OF BACITRACIN

Ointment Base	Zone of Inhibition Produced by the Ointments (mm.) Age of Ointments			Bacitracin Potency u./Gm. of Ointment Age of Ointments		
	1 wk.	2 mo.	6 mo.	1 wk.	2 mo.	6 mo.
Diester	7.5	6.9	6.0	530	..[a]	350
Jelene	4.6	4.4	4.2	480	..[a]	460
White petrolatum	3.0	2.4	2.0	500	..[a]	450
Atlas cream	7.0	3.3	0	..[b]	..[b]	..[b]
Tegacid	5.4	5.0	3.5	..[b]	..[b]	..[b]
Carbowax®	8.0	7.2	4.0	450	0[c]	..[a]
Span® 20	4.8	3.7	..[a]	400	200	..[a]
Hydrophilic petrolatum	4.7	3.6	..[a]	500	440	..[a]

[a] Not Tested. [b] No zones obtained at any time. [c] Less than 200 u./Gm. of ointment.

ments showed that the quantity of ointment on the disk made no difference provided the surface of the disk was covered and no ointment protruded beyond the edge of the disk.

Ointment Bases.—Of the different ointment bases tested as vehicles for bacitracin, the following may be taken as typical examples:

(*A*) *The diester of Jeffries, et al.*—Liquid petrolatum, 10.0 Gm.; White petrolatum, 10.0 Gm.; Glycowax S-932,[2] 10.0 Gm.; polyethylene glycol 400 distearate, 10.0 Gm.; polyethylene glycol 400 dilaurate, 60.0 Gm.

(*B*) *Jelene.*—A combination of mineral oils and heavy hydrocarbon waxes gelled by a special process, and having an average molecular weight of 1300 (4).

(*C*) *White Petrolatum, U. S. P.*

(*D*) *Atlas Cream Base.*—Stearic acid, 14.0 Gm.; White wax, 2.0 Gm.; Liquid petrolatum, 1.0 Gm.; Atlas G-2152[3] (polyoxyethylene stearate), 15.0 Gm.; distilled water, 50.0 Gm.

(*E*) *Tegacid Base.*—Tegacid,[4] 15.0 Gm.; Glycerin, 3.0 Gm.; spermaceti, 5.0 Gm.; methylparaben, 0.2 Gm.; distilled water, 60.0 Gm.

(*F*) *Carbowax Base.*—Carbowax® 4000,[5] 45.0 Gm.; propylene glycol, 55.0 Gm.

(*G*) *Span® 20 Base.*—Span® 20,[6] 5.0 Gm.; Wool fat, 20.0 Gm.; White petrolatum, 75.0 Gm.

(*H*) *Hydrophilic Petrolatum, U. S. P.*

After the bases had been prepared, stock bacitracin ointments containing 6,250 units of bacitracin per Gm. were prepared with each base. These stock ointments were used to prepare the remainder of the bacitracin ointments, all of which contained 500 units of bacitracin per Gm. They were placed in collapsible tin tubes and stored at room temperature.

Table I shows the zones of inhibition produced by bacitracin in the various ointment bases, and the bacitracin potency as determined by the extraction procedure of the F. D. A. None of the bases without bacitracin exhibited any evidence of inhibition.

To determine the effect of various individual components of ointment bases on the activity of bacitracin, these substances were added to either Jelene or white petrolatum in which bacitracin was incorporated. Table II shows the effect of various substances on the activity of bacitracin in white petrolatum. Table III shows the effect of various substances on the activity of bacitracin in Jelene. The control ointments, containing no bacitracin, yielded no evidence of inhibition with the exception of those containing sodium lauryl sulfate, cetylpyridinium chloride, and benzalkonium chloride; these ointments gave very faint, poorly-defined

TABLE II.—EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF BACITRACIN IN WHITE PETROLATUM

Substance Added to White Petrolatum		Zone of Inhibition Produced by the Ointments (mm.) Age of Ointments			Bacitracin Potency u./Gm. of Ointment Age of Ointments	
		1 wk.	2 mo.	4 mo.	1 wk.	4 mo.
None		3.0	2.3	2.0	500	450
White wax	3%	2.9	2.2	1.8	480	460
White wax	5%	2.9	2.2	1.8	480	450
White wax[a]	10%	2.9	2.3	2.0	..[b]	450
White wax[a]	20%	3.1	2.4	2.1	..[b]	470
Cetyl alcohol	3%	2.9	2.3	1.8	500	450
Stearyl alcohol	3%	3.1	2.3	1.5	480	420
Cholesterol	3%	2.5	1.3	T[c]	480	400

[a] Enough liquid petrolatum added to maintain consistency. [b] Not assayed. [c] T = trace of inhibition; zone too small to measure.

[2] Product of Glyco Products Company, Inc., Brooklyn, N. Y.
[3] Product of Atlas Powder Company, Wilmington, Del.
[4] Product of Goldschmidt Chemical Corporation, New York.
[5] Product of Carbide and Carbon Chemicals Company, New York.
[6] Product of Atlas Powder Company, Wilmington, Del.

TABLE III.—EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF BACITRACIN IN JELENE

Substance added to Jelene	Zone of Inhibition Produced by the Ointments (mm.) Age of Ointments 1 wk.	1 mo.	4 mo.
None	4.6	4.4	4.2
Wool fat 3%	4.5	4.2	4.0
Wool fat 5%	4.5	4.2	4.0
Wool fat 10%	4.5	4.2	4.1
Glycerin 5%	5.3	3.8	3.0
Glycerin 10%	5.7	4.0	3.3
Propylene glycol 5%	5.3	2.9	1.6
PEG 400[a] 5%	5.0	2.0	0.5
PEG 400[a] 10%	5.3	2.6	1.5
PEG 400[a] monostearate 1%	4.5	3.0	1.3
Span® 20 1%	6.1	5.8	4.3
Span® 65 1%	3.8	3.8	3.7
Tween® 61 1%	4.6	4.6	3.8
Tween® 80 1%	4.5	3.8	3.4
Sodium lauryl sulfate 1%	4.5	4.6	4.1
Aerosol® OT 0.1%	4.8	4.0	4.0
Cetylpyridinium chloride[b] 0.1%	5.1	4.3	3.5
Benzalkonium chloride 0.1%	5.1	4.3	2.7
Hydroquinone 2%	4.7	4.5	4.1
Hydroquinone 5%	5.0	5.0	4.3
Ascorbyl palmitate 2%	4.3	4.3	4.0
Ascorbyl palmitate 5%	4.0	4.0	3.8

[a] Polyethylene glycol 400. [b] Aqueous concentrate used.

zones of inhibition which were too small to measure.

To determine the effect of some antiseptics on the activity of bacitracin, various antiseptic ointments were prepared in each of three bases. Bacitracin was incorporated with part of each ointment in the concentration of 500 u./Gm., and the remainder used as a control. Table IV shows the inhibition zones produced by the ointments. Table V shows the bacitracin potency of the ointments after storage for six months. Ammoniated mercury ointment was the only ointment containing no bacitracin that showed any evidence of inhibition after extraction. This inhibition was expressed in terms of bacitracin potency.

DISCUSSION OF RESULTS

Table I shows that bacitracin was reasonably stable in the diester base, in Jelene, and in white petrolatum. White petrolatum released the drug very slowly as shown by the small zones of inhibition. The addition of stearyl alcohol and cholesterol to prepared hydrophilic petrolatum increased the size of the inhibition zone but slowly destroyed the activity of bacitracin. Attempts to assay the Tegacid and Atlas cream base met with failure; no inhibition zones were obtained even from extracts of freshly prepared ointments.

Table II shows that the addition of various per-

TABLE IV.—EFFECT OF VARIOUS ANTISEPTICS ON THE ACTIVITY OF BACITRACIN IN THREE OINTMENT BASES

Antiseptic	Zone of Inhibition Produced by the Ointments (mm.) Age of Ointments — 1 week Ointment Base A	B	C	6 weeks Ointment Base A	B	C	6 months Ointment Base A	B	C
Bacitracin—500 u./Gm.	7.5	4.6	3.0	7.0	4.4	2.3	6.5	4.2	2.0
Ammoniated mercury 5%	7.5	5.7	2.5	6.5	4.6	1.9	7.0	5.0	2.2
Ammoniated mercury and bacitracin	7.7	5.7	3.8	7.2	5.0	3.0	7.3	5.1	3.5
Boric acid 10%	5.2	0	0	5.0	0	0	4.9	0	0
Boric acid and bacitracin	9.0	6.0	1.5	7.0	5.5	0.8	6.5	5.0	T[a]
Benzoic and salicylic acids 12% and 6%	6.8	6.2	2.1	5.5	5.0	2.0	5.3	5.0	2.0
Benzoic and salicylic acids and bacitracin	8.3	6.2	2.9	6.3	5.6	2.5	5.4	5.6	2.0
Calamine 17%	0	0	0	0	0	0	0	0	0
Calamine and bacitracin	7.3	5.5	3.7	6.2	4.8	3.5	5.8	4.6	3.2
Ichthammol 10%	0	0.8	0	0	T	0	0	0	0
Ichthammol and bacitracin	1.0	3.3	2.2	0	2.2	1.7	0	0.6	1.6
Mercury bichloride 0.1%	5.5	2.3	T[a]	4.8	2.2	T[a]	5.0	2.2	T[a]
Mercury bichloride and bacitracin	7.3	5.0	2.4	5.0	4.5	2.0	5.0	4.3	0.8
Phenol 2%	0	0	0	0	0	0	0	0	0
Phenol and bacitracin	5.1	4.5	3.1	1.5	2.0	T[a]	0	0	0
Sulfathiazole 5%	0	0	0	0	0	0	0	0	0
Sulfathiazole and bacitracin	8.4	5.5	2.1	6.5	5.0	2.0	4.5	4.8	T[a]
Zinc oxide 20%	0	0	0	0	0	0	0	0	0
Zinc oxide and bacitracin	8.0	5.0	3.0	6.5	4.5	3.0	5.5	4.3	2.9
Coal tar 5%	0	0	0	0	0	0	0	0	0
Coal tar and bacitracin	7.5	5.3	1.5	7.0	4.1	1.0	5.0	4.0	T[a]
Resorcinol[b] 6%	0	0	0	0	0	0	0	0	0
Resorcinol and bacitracin[b]	6.8	5.5	2.3	6.0	3.1	1.3	5.5	1.0	0
Tannic acid 20%	1.0	3.3	3.0	T[a]	3.3	3.0	T[a]	2.7	2.7
Tannic acid and bacitracin	8.0	3.3	3.0	6.0	3.3	3.0	3.8	2.7	2.5
Sulfur 10%	0	0	0	0	0	0	0	0	0
Sulfur and bacitracin	9.0	5.0	2.4	6.5	4.2	2.6	3.5	3.8	1.0
Ethyl aminobenzoate 5%	0	0	..[d]	0	0	..[d]	..[c]	..[c]	..[d]
Ethyl aminobenzoate and bacitracin	7.0	4.0	..[d]	6.5	3.9	..[d]	..[c]	..[c]	..[d]

[a] Trace of inhibition; zone too small to measure. [b] Ointment turned bluish green within six weeks. [c] Ointment not tested [d] No ointment made.

TABLE V.—BACITRACIN POTENCY IN THREE OINTMENT BASES CONTAINING SOME ANTISEPTIC DRUGS AFTER SIX MONTHS STORAGE

Ointment	Bacitracin—u./Gm. of Ointment—Ointment Base— A	B	C
Bacitracin (fresh)	530	480	500
Bacitracin (6 months old)	350	460	450
Ammoniated mercury	640	475	400
Ammoniated mercury and bacitracin	640	450	400
Boric acid and bacitracin	0	450	360
Benzoic and salicylic acids and bacitracin	0	0	0
Calamine and bacitracin	300	450	300
Ichthammol and bacitracin	0	0	0
Mercury bichloride and bacitracin	T[a]	430	360
Phenol and bacitracin	0	0	0
Sulfathiazole and bacitracin	0	465	310
Zinc oxide and bacitracin	250	225	225
Coal tar and bacitracin	200	0	0
Resorcinol and bacitracin	200	0	0
Tannic acid and bacitracin	0	0	0
Sulfur and bacitracin	0	425	300
Ethyl aminobenzoate and bacitracin (2 months old)	435	435	..[b]

[a] Trace of inhibition; zone too small to determine potency.
[b] No ointment made.

centages of white wax to white petrolatum had no effect on the activity of bacitracin. Bacitracin potency assays showed that the ointments retained a potency of approximately 450 units per gram, yet the inhibition zones had decreased almost one-fourth. Cetyl alcohol appeared to have no effect, but stearyl alcohol, and to a greater extent, cholesterol, slowly inactivated the antibiotic.

Table III shows that the addition of various percentages of wool fat, hydroquinone, and ascorbyl palmitate to Jelene had no effect on the activity as measured by the inhibition zones produced by the ointments. Several of the surfactants had little or no effect on the activity. When first added, most of the surfactants slightly increased the inhibition zones. The increases were small and no appreciable advantage appeared to be gained by their addition.

Table IV shows that various substances acted in different ways on bacitracin in the different bases as determined by the sizes of the inhibition zones. For example, when first added, sulfathiazole and bacitracin had an additive effect in bases A and B, but an antagonistic effect in base C. After six months, smaller zones were obtained in bases A and C than were obtained from bacitracin in these bases. In base B, the zones were about the same size. Other minor variations were found. After storage for six months, bacitracin was found to be reasonably stable in the presence of calamine and zinc oxide in all three bases, and in the presence of boric acid, sulfathiazole, and sulfur in base B. The mixture of each drug with bacitracin in each base must be investigated separately to determine the effect on the activity of bacitracin.

Table V shows that bacitracin was reasonably stable in all three bases. In base A, boric acid inactivated the antibiotic. Sulfathiazole, mercury bichloride, and also sulfur apparently destroyed the activity in base A. Bacitracin was relatively stable in the three bases in the presence of calamine and zinc oxide. Benzoic and salicylic acids, ichthammol, phenol, coal tar, resorcinol, and tannic acid destroyed the activity in each base.

SUMMARY

The stability and relative availability of bacitracin in various ointments and when mixed with various substances in ointment bases were determined. There was limited correlation between bacitracin potency as determined by a modified F. D. A. method and the antiseptic efficacy of the ointments as measured by the agar plate diffusion method. The stability of bacitracin in anhydrous grease bases was confirmed. Bacitracin was found to retain most of its potency up to six months when mixed with the following substances in ointments:

Jelene	Hydroquinone
White petrolatum	Ascorbyl palmitate
Liquid petrolatum	Cetyl alcohol
White ointment	Calamine
White wax	Zinc oxide
Diester base	Ethyl aminobenzoate
Dried wool fat	

The following substances were found to destroy slowly the activity of bacitracin. These substances could be incorporated with bacitracin in ointments if the products were used within a few weeks, but no advantage appeared to be gained by their addition.

Stearyl alcohol	Span® 65
Cholesterol	Tween® 61
Span® 20	Tween® 80
Span® 20 base	Aerosol® OT
Hydrophilic petrolatum	Sodium lauryl sulfate
Carbowax® bases No. 1 and 2	

The following substances rapidly inactivated bacitracin in ointments:

Water	Glycerin
Water-containing bases	Ceepryn® chloride solution
Polyethylene glycol 400	Zephiran® chloride solution
Polyethylene glycol 400 monostearate	Ichthammol
	Phenol
Propylene glycol	Tannic acid

Since in the present investigation the results varied in the different ointment bases, each of the following substances must be considered in relation to each base to determine its effect on the activity of bacitracin:

Boric acid	Benzoic and salicylic acids
Sulfathiazole	Coal tar
Sulfur	Mercury bichloride
Ammoniated mercury	Resorcinol

Bacitracin exhibited greatest stability in Jelene.

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The Stability of Almond and Corn Oils for Use in Parenteral Solutions*

By RAFAEL P. HIZON and C. LEE HUYCK

The effect of autoclaving and storage under incubator temperature as well as the antioxygenic action of DBPC (2,6-di-tert-butyl-*p*-cresol) and propyl gallate on almond and corn oils was determined by measuring the peroxide value colorimetrically and the acid value volumetrically at intervals of two weeks. Of the oils tested, almond was found to be less stable than corn oil to the action of heat.

THE DEVELOPMENT of rancidity in fats and oils as a result of atmospheric oxidation is a major problem in the food and drug industry. The resultant effects of such normal oxidative deterioration in drugs are the development of undesirable odors and tastes with an increase in toxicity. A very important aspect of this problem is the development of excessive acidity in oils used as solvents for drugs administered parenterally since acidity over 0.1% cannot be used (1).

The stability of almond and corn oils as oils for injection was determined under accelerated conditions of temperature with and without the addition of 2,6-di-tert-butyl-para-cresol (DBPC[1]) and propyl gallate[2] as antioxidants.

In order to evaluate the antioxidant properties of these various substances and to be able to follow the progress of antioxidation of stored oil samples, several quantitative test methods for determining their peroxide content were proposed as follows: (*a*) modification of the Lea's iodometric method (2), (*b*) modification of the Swift stability test (3), (*c*) the Kries test (4) so modified (5, 6) that the degree of rancidity is determined by measuring the pink or red color produced from the condensation of epihydrin aldehyde and phloroglucinol by a spectrophotometer.

The method of determining rancidity used in this study is the method recently published by Golden (9). Peroxides in fats and oils are determined from the oxidation of freshly prepared colorless ferrous thiocyanate to red ferric thiocyanate having an intensity of color in proportion to the amount of peroxide present. Since the light absorption of this system follows Beer's law, the spectrophotometric method is applicable.

EXPERIMENTAL

Materials.—Sufficient quantities of sweet almond and refined corn oils without preservative or antioxidant were purchased and used as examples of oils for injection.

The 2,6-di-tert-butyl-para-cresol and propyl gallate were used as antioxidants for the fixed oils. The DBPC was labeled "food grade," and had the following specifications (7):

Purity, min. (by freezing point)	98.7
Freezing point, °C. min.	69.2
Color of molten mass, Hellige, max.	2.0
Solubility: per cent by weight at 20°C.	
Mineral oil	30.0%
Linseed oil	28.0%
Water	insoluble
10% NaOH	insoluble

The test reagent used was freshly prepared ferrous thiocyanate. Nitrogen for technical use was employed in the preparation of this reagent.

A three-necked, round-bottomed flask with reflux condenser, syphon for convenient removal of the test solution, and attachment for nitrogen gas was used for the preparation of the ferrous thiocyanate.

A Coleman Junior spectrophotometer, Model 6, Inst. N. a-330 was used in the determination of peroxide value by measuring the absorbancy of the oxidized ferrous thiocyanate solution. The cell adapter was modified to accommodate Pyrex test tubes that were optically matched and calibrated to indicate volumes of 5 and 12 cc. A Kodak stop clock for controlling the length of time to mix the test reagent and the sample and the time interval be-

* Received May 5, 1955, from the St. Louis College of Pharmacy and Allied Sciences, St. Louis 10, Mo.
[1] DBPC is the trade name of Koppers Company, Inc. (Chemical Division), Pittsburgh, Pa., for 2,6-di-tert-butyl-para-cresol.
[2] Propyl gallate was furnished through the courtesy of Goldschmidt Chemical Corporation, New York, and Fine Organics, Inc., New Jersey.

tween mixing and the spectrophotometric readings, a cylinder of nitrogen gas to protect the sample and solvent during assay, and a constant temperature bath to maintain a temperature of 25° during determination of peroxide values, were also employed.

Preparation of Samples and Subsequent Storage.—A fresh sample of sweet almond oil free from antioxidants was divided into three portions. To the first portion no antioxidant was added, to the second portion, 0.02% DBPC was added and mixed thoroughly until dissolved. To the third portion, 0.02% propyl gallate was incorporated and the mixture shaken until the added antioxidant was completely dissolved.

Three 6-fluid ounce amber bottles were completely filled with the first portion and stoppered securely. One of the bottles was stored in a refrigerator at temperature of 9° to 11°, another was kept at room temperature of 20–28°, and the third was immediately autoclaved at 16 pounds pressure (122°) for 30 minutes, after which it was stored in an incubator heated to 37.5°. The second and third portions which contain the antioxidants were each poured into three 6-fluid ounce amber bottles and each series was treated and stored as oultined for the oils without antioxidant. This made a total of nine test samples for each oil. In order to prevent errors the samples were numbered chronologically as follows:

	Almond Oil	
1.	Without antioxidant	Refrigerated
2.	Without antioxidant	Stored at room temperature
3.	Without antioxidant	Autoclaved and incubated
4.	With 0.02% DBPC	Refrigerated
5.	With 0.02% DBPC	Stored at room temperature
6.	With 0.02% DBPC	Autoclaved and incubated
7.	With 0.02% propyl gallate	Refrigerated
8.	With 0.02% propyl gallate	Stored at room temperature
9.	With 0.02% propyl gallate	Autoclaved and incubated
	Corn Oil	
10.	Without antioxidant	Refrigerated
11.	Without antioxidant	Stored at room temperature
12.	Without antioxidant	Autoclaved and incubated
13.	With 0.02% DBPC	Refrigerated
14.	With 0.02% DBPC	Stored at room temperature
15.	With 0.02% DBPC	Autoclaved and incubated
16.	With 0.02% propyl gallate	Refrigerated
17.	With 0.02% propyl gallate	Stored at room temperature
18.	With 0.02% propyl gallate	Autoclaved and incubated

Assay Procedures.—Directly after preparation of the samples they were assayed for acid and peroxide values at intervals of two weeks and then returned to their respective storage conditions. This was done in order to detect the onset of rancidity and to evaluate the added compounds with respect to their antioxidant properties; also to follow the progress of their autoxidation or deterioration.

Acid Value.—The acid value is defined as the number of mg. of potassium hydroxide required to neutralize the free fatty acids in one gram of sample. In this investigation, the acid values of the samples of oils for injection were determined in accordance with the general procedure given in the National Formulary (8).

The 0.1 *N* sodium hydroxide was standardized against reagent potassium biphthalate which was previously dried at 100° for three hours. The normality of the standard alkali solution was checked against a standard hydrochloric acid solution every time it was used in the determination of the acid value of the samples. The results of the acid value determinations are shown in Table I.

Peroxide Value.—The peroxide value was determined colorimetrically using a Coleman Junior spectrophotometer following the method of Golden (9).

Materials.—Ferrous sulfate C. P., ammonium thiocyanate C. P., iron powder reduced by hydrogen N. F., iron wire for standardization N. F., acetone U. S. P., ethyl ether[3] U. S. P., light mineral oil[4] N. F., standardized hydrogen peroxide solution.[5]

Preparation of the Test Reagent.—In a liter round-bottomed wide-mouthed flask, 3 Gm. of reagent ferrous sulfate was dissolved in 150 cc. of distilled water to which 3 cc. of concentrated sulfuric acid was added. Three hundred cc. of U. S. P. acetone and 150 cc. of 2% aqueous solution of reagent ammonium thiocyanate were then added to the flask. Iron powder and iron wire in the amount of 0.3 Gm. and 9 inches, respectively, were finally incorporated into the mixture. The resulting solution was red in color due to the presence of ferric ions. The mixture was then refluxed by heating on a hot plate for at least two hours or until all traces of any pink color had disappeared and the resulting test solution of ferrous thiocyanate was colorless. During refluxing the red ferric ions were reduced to colorless ferrous ions by the nascent hydrogen produced by the action of the acid on the iron powder and wire.

At this step in the procedure, nitrogen gas protection was begun and the heat discontinued. This precaution eliminated the possibility of absorption of atmospheric oxygen. Since it was important to avoid contact with air, the solution was dispensed directly from the flask by means of the syphon which was initially operated by the pressure developed by the inert gas on the surface of the liquid.

This test solution was freshly prepared each day that a determination was made. The amounts specified were sufficient to prepare a quantity of solution that was adequate for 30 peroxide-value determinations.

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[3] Obtained from St. Louis Division, E. R. Squibb & Sons.
[4] Obtained from Rexall Drug Co., St. Louis, Mo.
[5] Used Superoxol from Merck & Co.

TABLE I.—EFFECT OF ADDED ANTIOXIDANTS AND ACCELERATED TEMPERATURE CONDITIONS ON ACID[a] AND PEROXIDE[b] VALUES

Storage conditions		Initial	Without Antioxidant 2 wks.	4 wks.	6 wks.	8 wks.	10 wks.	Initial	With 0.02% "DBPC" 2 wks.	4 wks.	6 wks.	8 wks.	10 wks.	Initial	With 0.02% Propyl Gallate 2 wks.	4 wks.	6 wks.	8 wks.	10 wks.
Almond Oil																			
Refrigerated[c]	a	.095	.126	.120	.108	.120	.115	.120	.107	.088	.114	.133	.121	.126	.145	.164	.145	.159	.166
	b	24.6	26.9	24.6	51.4	52.0	52.0	22.1	26.9	23.5	37.7	43.3	43.4	22.1	28.0	30.9	32.1	38.5	40.7
Room temp.[d]	a	.095	.095	.107	.107	.120	121	.120	.095	.095	.094	.114	.127	.126	.126	.152	.158	.178	.172
	b	24.6	26.9	39.1	46.4	47.0	54.7	22.1	28.0	37.7	34.9	46.3	45.1	22.1	24.2	37.2	48.6	44.5	46.3
Autoclaved and incubated[e]	a	.206[f]	.145	.133	.140	1.59	.185	.285	.094	.133	.127	.152	.179	.190	.151	.158	.171	.178	.192
	b	19.2[f]	33.4	37.8	82.3	100.5	118.2	20.8	25.1	48.9	51.9	63.9	72.6	13.7	22.4	38.5	40.7	46.3	46.3
Corn Oil																			
Refrigerated[c]	a	.139	.045	.038	.069	.082	.070	.095	.052	.044	.076	.082	.083	.095	.095	.101	.088	.114	.127
	b	21.0	14.3	24.0	26.3	32.7	34.9	19.1	9.3	24.5	27.9	33.2	32.7	14.9	15.5	20.7	23.4	29.6	29.1
Room temp.[d]	a	.139	.158	.088	.076	.070	.070	.095	.063	.050	.063	.082	.089	.095	.126	.095	.101	.120	.127
	b	21.0	30.8	19.4	22.4	37.7	39.5	19.1	26.3	32.0	37.6	39.8	40.5	14.9	15.5	26.3	29.6	39.7	39.4
Autoclaved and incubated[e]	a	.221[f]	.062	.044	.069	.089	.095	.158	.050	.057	.069	.082	.083	.190	.094	.095	.095	.127	.133
	b	7.8[f]	10.4	32.1	32.1	38.4	40.6	7.9	26.8	43.3	46.2	47.7	48.5	13.6	11.7	32.0	37.6	38.9	39.4

[a] Expressed in mg. of KOH per Gm. of oil. [b] Expressed in mg. of H_2O_2 per Kg. of oil. [c] Stored in a refrigerator at temperature range of 9 to 11°C. [d] Stored at 22 to 28°C. [e] Immediately sterilized under 16 lbs. pressure at 122°C. and then stored in an incubator at 37.5°C. [f] Determined immediately after autoclaving.

Standardization Curve.—From a concentrated hydrogen peroxide solution a dilute solution of about 2% concentration was prepared. This was assayed volumetrically following the U. S. P. XIV method using potassium permanganate solution that had been previously standardized against reagent sodium oxalate heated to constant weight at 110°.

A series of ten dilutions were then made from the assayed hydrogen peroxide solution as follows:

No.	Contained H_2O_2 per cc.	mg./0.1 cc.
1	0.01 mg.	(1×10^{-3})
2	0.02 mg.	(2×10^{-3})
3	0.03 mg.	(3×10^{-3})
4	0.04 mg.	(4×10^{-3})
5	0.05 mg.	(5×10^{-3})
6	0.06 mg.	(6×10^{-3})
7	0.07 mg.	(7×10^{-3})
8	0.08 mg.	(8×10^{-3})
9	0.09 mg.	(9×10^{-3})
10	0.10 mg.	(1×10^{-2})

The absorbance of the dilute hydrogen peroxide solutions was determined by means of the spectrophotometer and calibrated accessories previously described. Exactly 0.1 cc. of the solutions accurately measured in 0.1 cc. volumetric pipets, were used in the determinations which will be described in more detail under *Test Method*. All spectrophotometric readings were made at 560 mμ wavelength. A blank determination was carried out and the reading obtained was deducted from the reading of the test to give the absorbance due to hydrogen peroxide.

The peroxide concentration was then plotted against absorbance and the resulting standardization curve used in the calculation of peroxide values. As shown in Fig. 1, the colored solution was found to obey Beer's law at concentration range of 1×10^{-3} to 5×10^{-3} mg./cc. At higher concentrations, although the curve continued to be a straight line, its slope was moderately depressed.

Test Method.—A volume of 0.1 cc. of the oil sample accurately measured in a 0.1 cc. volumetric pipet was transferred to the specially-calibrated test tubes. These were closely matched in the Coleman Junior spectrophotometer and calibrated to indicate the 5-cc. and 12-cc. volume. Enough solvent consisting of 25% ethyl ether w/v in light mineral oil was then added to bring the volume to the 5-cc. mark. Technically pure nitrogen gas was immediately bubbled through the mixture for exactly one minute. The test tube was stoppered tightly to avoid contact with the air. A sufficient amount of the colorless ferrous thiocyanate test solution was quickly introduced into the tube by means of the syphon dispenser until its level reached the 12-cc. mark. The stopper was immediately replaced and the mixture was shaken gently for exactly 30 seconds. The tube was allowed to stand until the lower layer of test solution separated into a clear aqueous phase.

In the meantime another matched test tube containing distilled water was placed in the Junior Coleman spectrophotometer, set to wavelength of 560 mμ and the absorbance scale adjusted to zero. The tube of distilled water was then replaced by the tube containing the sample and the test solution. After exactly two minutes, the amount of pink or red color formed was measured by recording the ab-

sorbance. A blank determination (without oil sample) was carried out simultaneously to obtain the absorbance due to the ether-mineral oil mixture and the test solution. The difference in the scale reading between the sample and the blank gave the absorbance, due to peroxide present in 0.1 cc. of oil.

The amount of peroxide oxygen expressed as mg. of hydrogen peroxide per Kg. of sample was taken as the peroxide value. The weight of the samples used was calculated from the specific gravity of the oil determined at 25° by the use of a pycnometer. All the samples were maintained at 25° in a constant temperature bath before peroxide value was determined. The specified time intervals were strictly adhered to in all the determinations.

From the results obtained in this method, the peroxide values of the samples were easily computed using the standardization curve as follows:

The absorbance obtained was first converted to milligrams of hydrogen peroxide by means of the standardization curve. From this hydrogen peroxide equivalent the peroxide value may be calculated using this formula:

$$\text{Peroxide value} = \text{mg. } H_2O_2 \times \frac{1000}{\text{vol. of sample at } 25^\circ \times \text{sp. gr. of sample at } 25^\circ}$$

The results of the peroxide value determinations are shown in Table I. The peroxide values for Table I were then plotted against time in order to evaluate the effect of heat and the added antioxidants on the rate of autoxidation of the samples of oils. This relationship is shown graphically in Fig. 2, 3, 4, and 5.

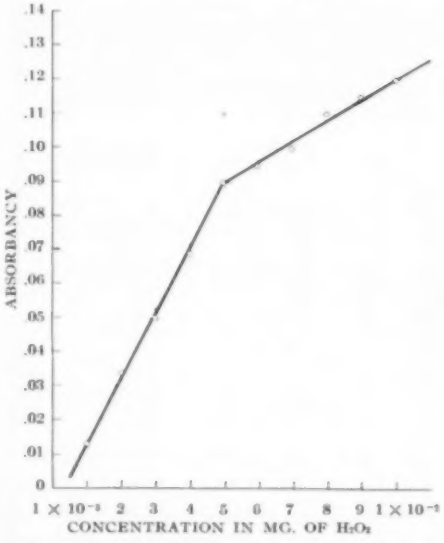

Fig. 1.—Standardization curve showing relationship between the concentration of hydrogen peroxide and absorbance.

DISCUSSION OF RESULTS

Table I shows that the rate of autoxidation of almond oil was accelerated by an increase in temperature. The peroxide value was lowered from 24.6 to 19.2, while the acid value was increased from 0.095 to 0.296 after autoclaving at 122° for 30 minutes. Subsequent storage at 37.5° caused a rapid increase in peroxide value and a corresponding decrease in acid value. On the tenth week, the sample that was autoclaved and stored in the incubator showed a peroxide value of 118.2 compared to 52 and 47 for samples which were stored at refrigerator and room temperatures, respectively.

The peroxide value of corn oil upon autoclaving was lowered from 21.0 to 7.8 while its acid value increased from 0.139 to 0.221. On the tenth week, the peroxide value went up to 40.6 which was approximately of the same level as the peroxide value of the samples stored at room and refrigerated temperatures. The decrease in peroxide value and the resulting increase of acid value on heating the oils was in agreement with the finding of Dzens (10) and explained by Eckey (11) as being due to secondary reactions in which the hydroperoxides present react to form a variety of other oxidation products such as acids and aldehydes.

The results obtained from peroxide value determinations on almond and corn oils stored at refrigerator, room, and incubator temperatures clearly show that corn oil is more stable to heat than almond oil. In similar experiments Kedvessy (12) also observed that almond oil seemed to become rancid more quickly than most fixed oils. According to Eckey (11), this heat-stability-characteristic of corn oil is due to the presence of natural antioxidants or inhibitors, the most important of which is the tocopherol occurring in corn oil to the extent of 0.12%. Figures 2 and 4 show the comparative effect of heat on almond and corn oils.

The effect of added antioxidants on the rate of peroxide formation in almond and corn oils is shown in Table I. The results indicate that "DBPC" and propyl gallate exert marked antioxygenic action and prolong the induction period of almond oil, while the antioxidant effect on corn oil is negligible. It seems that the presence of natural antioxidants has no bearing on the initial oxidation and induction period but shows up later. It was expected that the antioxidant effect of "DBPC" and propyl gallate on corn oil would become more pronounced as autoxidation progresses.

In the case of almond oil which contained no added antioxidant and stored at 37.5°, the peroxide value after ten weeks rose to 118.2 as compared to 72.6 and 46.3 for samples containing 0.02% "DBPC" and propyl gallate, respectively. In corn oil the same concentration of the antioxidant failed to exhibit appreciable protection, since the peroxide value of the control and test samples were found to be approximately at the same level after ten weeks of incubator temperature. Figures 3 and 5 illustrate the effect of the presence of 0.02% "DBPC" and of 0.02% propyl gallate in almond and in corn oil.

A comparison of the antioxidant effect of propyl gallate and "DBPC" seemed to indicate that the former affords greater protection to both almond and corn oil. Data from Table I show that after ten weeks, the peroxide value of the sample of al-

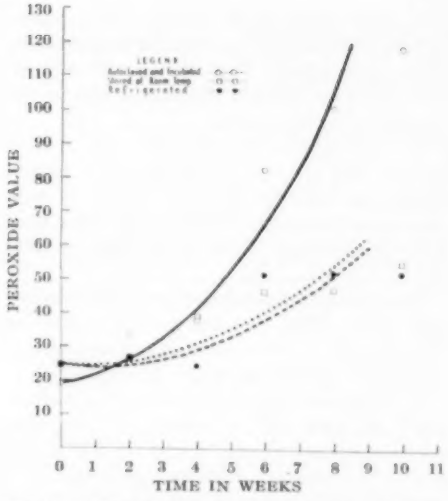

Fig. 2.—Effect of storage temperature on the peroxide value of almond oil.

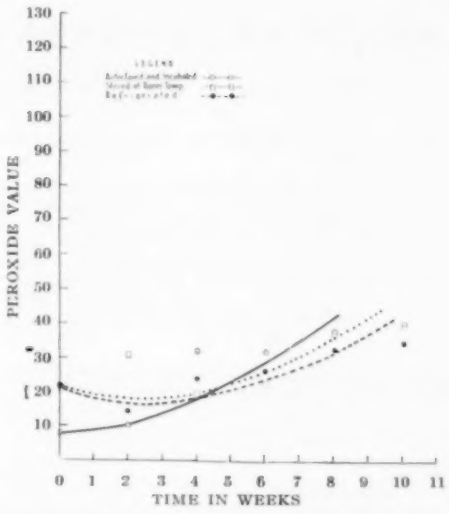

Fig. 4.—Effect of storage temperature on the peroxide value of corn oil.

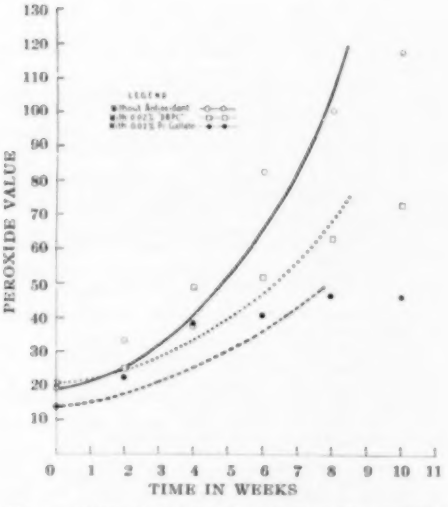

Fig. 3.—Effect of added antioxidants on the peroxide value of almond oil at incubator temperature.

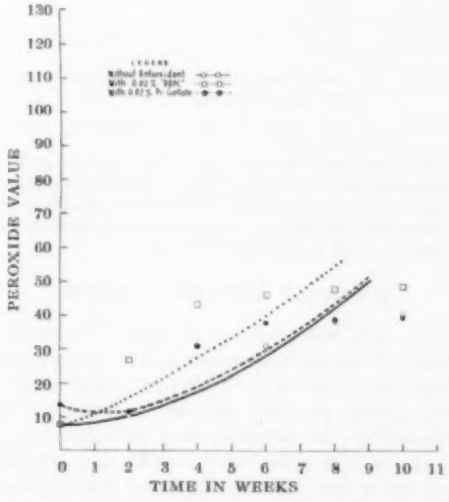

Fig. 5.—Effect of added antioxidants on the peroxide value of corn oil at incubator temperature.

mond oil containing 0.02% of "DBPC" and stored at 37.5° was 72.6 as contrasted with 46.3 for the same oil containing 0.02% propyl gallate. For corn oil, the peroxide values after ten weeks under the same storage condition were 48.5 and 39.4, respectively.

The acid value was not used as a measure of the degree of autoxidation of the oil samples because as Jamieson (13) emphasized, the presence of free fatty acids either in small or large quantities is no indication whatsoever of rancidity. In other words, rancid fats or oils may contain either small or large quantities of free fatty acids.

There exists a conflicting opinion among workers on the relationship of peroxide values to acid values in fixed oils. Razina (14) and Glimm (15) found that the increase in peroxide value is parallel to that of the acid value, while Jamieson (13) and Voillier (16) maintain that there is no relation between these values. An attempt was made to determine from the data in Table I the degree of correlation if any between the acid and peroxide value. Using the method and equations recommended by Goedicke (17) the following values were obtained:

$$\bar{v} = \frac{13}{108} = 0.12 \qquad \overline{v^2} = \frac{342}{108} = 3.17$$

$$\bar{u} = \frac{-79}{108} = -0.73 \qquad \overline{u^2} = \frac{149}{108} = 1.38$$

$$\overline{uv} = \frac{9}{108} = 0.083$$

$$u = \sqrt{1.38 - (-0.73)^2} = 0.92$$

$$v = \sqrt{3.17 - (0.12)^2} = 1.77$$

$$\text{Correlation Coefficient } (r) = \frac{\overline{uv} - \bar{u}\bar{v}}{u\,v}$$

$$r = \frac{0.083 - (-0.73 \times 0.12)}{0.92 \times 1.77} = 0.104$$

To test the significance of this correlation coefficient Rider (18) gives the following method:

$$X = 1/2 \log_e \frac{1 + r}{1 - r}$$

$$= 1.1513 \log_{10} \frac{1 + 0.104}{1 - 0.104}$$

$$X = 0.1075$$

$$x = \frac{1}{(N - 3)^{1/2}} = \frac{1}{(108 - 3)^{1/2}} = 0.097$$

$$t = \frac{X - 0}{x} = \frac{0.1075 - 0}{0.97} = 1.108$$

$A_t = 0.34326$ (obtained from table areas under the normal curve)

$$P = 1.00 - 2A = 1.00 - 0.6865 = 0.3135$$

This shows that the calculated value for the correlation coefficient is not significant. Therefore no relation between acid and peroxide values is established. These findings are in agreement with the findings of Jamieson (13) and Voillier (16).

SUMMARY AND CONCLUSIONS

1. The effect of autoclaving and storage under incubator temperature as well as the antioxygenic action of "DBPC" and propyl gallate on almond and corn oils were determined by measuring the peroxide value colorimetrically and the acid value volumetrically at intervals of two weeks. Of the oils tested almond was found to be less stable than corn oil to the action of heat. This leads to the conclusion that corn oil will withstand the heat sterilization process in the preparation of parenterals better than almond oil and will exhibit better keeping qualities than almond oil.

2. The antioxidants "DBPC" and propyl gallate were found to be effective in prolonging the induction period of almond oil. In corn oil, this protection was less pronounced due to the presence of natural antioxidants.

3. Comparison of the antioxidants used showed that at 0.02% concentration propyl gallate was the more effective inhibitor for both almond and corn oils. DBPC on the other hand had the advantage of greater solubility in fixed oils.

4. Statistical determinations of the relationship between the acid and peroxide values of almond and corn oils in the course of autoxidation revealed a very low coefficient of correlation.

5. The results of this investigation show that fixed oils employed as solvents for parenteral solutions possess varying degrees of stability to heat. To achieve a maximum shelf-life for a particular oil for injection, it is important that it contain an effective antioxidant.

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The Use of Cocoa Syrups for Masking the Taste of Quinine Hydrochloride*

By ARTHUR W. REID and CHARLES H. BECKER

Eleven different formulas of cocoa syrup were used to determine statistically which would best mask the taste of quinine hydrochloride. These were compared with cocoa syrup U. S. P., and syrup, U. S. P. Tests made to determine whether the addition of flavors or varying the method of preparation would improve the disguising power of cocoa syrups showed that ten of the eleven cocoa flavored vehicles prepared were significantly better disguising agents than the official cocoa syrup. Three syrups made with Dutch process cocoa showed the highest disguising potentials. All cocoa syrups tested were significantly better disguising agents than syrup, U. S. P.

WRIGHT (1) AND PURDUM (2) found that cocoa and eriodictyon syrups were powerful masking agents for quinine hydrochloride. Purdum later came to the conclusion that cocoa syrup was the best of the official vehicles for use with quinine. Entrekin (3) found cocoa syrup the best of all preparations tested for overcoming the bitterness of quinine hydrochloride. Lankford and Becker (4) also came to the conclusion that cocoa syrup is the best of all vehicles for disguising quinine hydrochloride and Boothe and Kaufman (5) showed that cocoa syrup is extremely effective for masking quinine sulfate. Narinian, Ohmart, and Stoklosa (6) have recently suggested a new formula for cocoa syrup which they consider equal to or better in flavor and stability to all other formulas used. However, in all these cases, no studies were made varying the method of preparation, nor were studies carried out to see if added substances, such as cocoa butter and peppermint oil, would enhance the disguising powers of cocoa syrups to mask better the taste of a distasteful drug. It was, therefore, the purpose of this investigation to evaluate these factors, using quinine hydrochloride as the distasteful drug.

EXPERIMENTAL

The materials and formulas of imitation concentrates and cocoa syrups used in this investigation were the same as described in a previous paper (7). The preparation of taste test samples and controls, as well as the method of evaluation of the vehicles used in this study were the same as described in a previous paper (8) with the modification indicated below.

TABLE I.—THE EFFECTIVENESS OF SOME COCOA FLAVORED AND OFFICIAL VEHICLES ON THE DISGUISING POTENTIALS FOR QUININE HYDROCHLORIDE

Vehicle	Number of Subjects	Mean Threshold in Water Standard Error $m_2 + e_2$	Mean Threshold in Vehicle Standard Error $m_1 + e_1$	Mean Disguising Potential Standard Error $D + e$	Significance of Difference
T_{12}	15	5.2 ± 0.38	16.3 ± 0.18	11.1 ± 0.43	26.592
T_4	15	4.7 ± 0.54	15.6 ± 0.31	10.9 ± 0.51	17.555
T_{13}	15	5.6 ± 0.54	16.4 ± 0.25	10.8 ± 0.52	18.048
T_{10}	15	4.7 ± 0.51	15.2 ± 0.40	10.5 ± 0.47	50.847
T_9	15	4.7 ± 0.51	15.1 ± 0.29	10.5 ± 0.52	17.627
T_5	15	4.3 ± 0.34	14.7 ± 0.21	10.4 ± 0.31	27.736
T_6	15	4.3 ± 0.34	14.5 ± 0.21	10.2 ± 0.34	25.095
T_3	15	4.7 ± 0.54	14.6 ± 0.37	9.9 ± 0.50	15.212
T_7	15	4.9 ± 0.50	14.3 ± 0.37	9.5 ± 0.59	14.942
T_8	15	4.9 ± 0.50	13.9 ± 0.34	9.1 ± 0.38	14.701
T_2	15	4.9 ± 0.36	13.5 ± 0.37	8.5 ± 0.64	16.853
T_1	15	4.8 ± 0.33	11.6 ± 0.64	6.8 ± 0.74	9.482
T_{11}	15	5.7 ± 0.52	9.5 ± 0.36	3.9 ± 0.46	5.969

The concentrations of quinine hydrochloride used here were the same as those used by Entrekin (3) except that higher concentrations, numbered 16, 17, and 18 were added. The normalities of those solutions were 0.00129, 0.00194, and 0.00291 resepectively, and each 4-cc. taste test sample, respectively contained 0.0021, 0.0032, and 0.0048 Gm.

The results in Table I indicate that syrup T_{12} (made from Dutch process cocoa by the hot method with salt added) had the highest potential for masking the bitter taste of the drug. It was followed closely by syrup T_4 (made from Dutch process cocoa by a cold method), syrup T_{13} (made from Dutch

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TABLE II.—THE SIGNIFICANCES OF DIFFERENCES BETWEEN THE MEAN DISGUISING POTENTIALS OF COCOA FLAVORED AND OFFICIAL VEHICLES IN TABLE I FOR QUININE HYDROCHLORIDE

T_{12} Cocoa syrup (Dutch), hot method with salt	T_4 Cocoa syrup (Dutch), cold with no flavor	T_{13} Cocoa syrup (Dutch), hot method with butterscotch	T_8 Cocoa syrup (American), hot method with salt	T_{10} Cocoa syrup, (American), hot method with imitation rum	T_9 Cocoa syrup (American), hot method with butterscotch	T_6 Cocoa syrup (American), hot method with peppermint oil	T_3 Cocoa syrup (American), cold method with no flavor	T_7 Cocoa syrup (American), cold method with skimmed milk	T_5 Cocoa syrup (American), hot method with cocoa butter	T_2 Cocoa syrup (Dutch), hot method with no flavor	T_1 Cacao Syrup, U. S. P.	T_{11} Syrup, U. S. P., hot method
.30												
.44	.14											
.89	.55	.41										
.94	.58	.43	0									
1.33	.84	.66	.17	.18								
1.64	1.14	.96	.49	.52	.44							
[a]1.81	1.40	1.24	.83	.88	.85	.49						
2.20	1.80	1.66	1.28	1.34	1.36	1.03	.52					
3.47	2.81	2.62	2.18	2.32	2.65	2.14	1.27	.57				
3.37	2.93	2.78	2.44	2.53	2.68	2.35	1.72	1.15	.80			
5.00	4.54	4.39	4.09	4.21	4.47	4.15	3.45	2.85	2.75	1.73		
11.47	10.21	9.93	9.59	10.11	11.83	11.05	8.84	7.55	8.72	5.86	3.32	0

[a] All values below this line indicate significant differences between the mean disguising potentials.

process cocoa by the hot method with imitation butterscotch added), syrup T_{10} (made from American process cocoa by the hot method with imitation rum flavor), syrup T_9 (made from American process cocoa by the hot method with salt added), and syrup T_8 (made from American process cocoa by the hot method with imitation butterscotch added).

All of the vehicles tested were significantly better than water as disguising agents for the bitter taste of quinine hydrochloride, the significance of difference for each comparison being always greater than 2.05. In the "null case" of no difference in disguising potential, sampling variation would give rise to a significance of difference as great as 2.05 in only 5% of the cases.

Table I also shows that the three syrups with highest disguising potentials were prepared from Dutch process cocoa. This may be due to the fact that Dutch process cocoa has a more bitter taste than American process cocoa which aids in masking the bitter taste of the drug and also to the higher fat content found in the Dutch process cocoa used.

The results in Table II indicate that ten of the eleven cocoa-flavored vehicles studied were significantly better than the official cocoa syrup for disguising the taste of quinine hydrochloride. All eleven were significantly superior to syrup, U. S. P., as disguising agents. These facts may be seen by noting which syrups obtained a value of 2.05 or greater when compared to the official cocoa syrup and to syrup, U. S. P.

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Composition of Gum Turpentines of Pines XXVI.*

A report on *Pinus lawsoni* and *P. herrerai* from Michoacán, Mexico, and *P. ponderosa* from the California Coast

By N. T. MIROV and P. M. ILOFF, Jr.

Pinus lawsoni **gum turpentine contained: 55% *d*- and *dl*-α-pinene; 31% *d*-Δ³-carene; 2–3% *l*- and *dl*-limonene; 2% terpinolene; 2% methyl chavicol and 5% sesquiterpene, almost entirely *d*-longifolene. Residue and losses, 2.6%. The gum turpentine of *P. herrerai* contains: *d*- and *dl*-α-pinene, 88%; *d*-Δ³-carene, 1–2%; dipentene, 0.5–1%; an unidentified oxygenated compound, 1%; sesquiterpenes, mostly *d*-longifolene, 2%; pot residue and loss, 6.7%. The gum turpentine from *P. ponderosa* from Santa Cruz consists of: 6% *l*- and *dl*-α-pinene; 43% *l*-β-pinene; 2% β-myrcene; 2% *l*-β-phellandrene (?) suspected but not identified; 25% *d*-Δ³-carene; 10% *l*- and *dl*-limonene; 0.2% undecane; 2.3% unidentified oxygenated compounds; 4.5% sesquiterpenes including a cadinene-like sesquiterpene; and residue and losses, 5.0%.**

PINUS LAWSONI

Pinus lawsoni Roezl is a subtropical pine of the Group *Australes*, growing in central and western Mexico (States of Jalisco, Michoacán, Morelos, México, Puebla, Guerrero, Oaxaca). A sample of oleoresin and herbarium specimens of this pine were collected by Ing. Mário Avila González near Ciudad Hidalgo, Michoacán,[1] at an elevation of 7,540 feet.

EXPERIMENTAL

Turpentine was obtained in the following manner. A batch of 4,815 Gm. of the oleoresin was heated under reduced pressure, so that towards the end of the distillation, the temperature was increased to 195° and pressure was reduced to 1 mm. of mercury. Yield of turpentine amounted to 20.8 per cent of the weight of the oleoresin. The remaining rosin was hard and brittle, indicating that all volatile ingredients had been removed from the oleoresin. The turpentine had the following physical properties: d_4^{22}, 0.8627; n_D^{21}, 1.4725; $[\alpha]^{22.5}_D$, +24.0.

A batch of 974 Gm. of *P. lawsoni* turpentine was fractionated with a 90-cm. long, 25-mm. inside-diameter Todd column, packed with 3/32-inch single-turn glass helices; a reflux ratio of 10 to 1 was maintained.

The physical properties of fraction 2 indicated that it was *d*- and *dl*-α-pinene; *dl*-α-pinene nitrosochloride was prepared from 7 ml. of fraction 2. After two recrystallizations from chloroform by addition of cold methanol, the derivative (0.5 Gm.) melted at 103.5–104.5°; its melting point was not depressed by addition of authentic *dl*-α-pinene nitrosochloride. Fractions 3 and 4 also contained mainly α-pinene.

Judging from the physical properties, fractions 5 to 10 were composed of varying amounts of α-pinene and Δ³-carene.

From 5 Gm. of oil from fraction 11, 502 mg. of Δ³-carene nitrosate was prepared. The derivative melted at 144–145°, and its melting point was not depressed by admixture with authentic Δ³-carene nitrosate.

Fractions 13 and 14 had properties indicating the possible presence of limonene. Fraction 14 was redistilled over sodium, yielding a heart cut with the properties: b^{760}, 175–176°; d_4^{23}, 0.8497; n_D^{19}, 1.4781; $[\alpha]^{23}_D$, −32.5°. A 1-ml. portion of the heart cut was used to prepare a tetrabromide which after three recrystallizations from ethyl acetate melted at 123–124°; its melting point was not depressed by admixture of authentic *dl*-limonene tetrabromide. The yield of tetrabromide was very small. Fraction 15 was similar in properties to fraction 14.

Fractions 17 and 18 were transition fractions. Fraction 18 had physical properties near those of terpinolene. This fraction was redistilled over sodium, yielding a heart cut with the following properties: b^{760}, 183–184°; d_4^{23}, 0.856; n_D^{20}, 1.4895; $[\alpha]^{23}_D$, −2.1°. A 2-Gm. portion of the heart cut was used to prepare a tetrabromide which, after several recrystallizations from chloroform by addition of cold methanol, yielded a few mg. of white crystals melting at 116–117°. Admixture with authentic terpinolene tetrabromide did not depress the melting point.

Fractions 19, 20, and 21 were transition fractions containing both terpenes and methyl chavicol. Fractions 22 and 23 had a high density and refractive index and an odor which indicated the presence of methyl chavicol. A 10-Gm. portion of the two fractions was oxidized at 0° with aqueous potassium permanganate acidified with acetic acid. After working up in the usual manner, 120 mg. of white crystals, m. p. 84–85°, was received. No depression in melting point was observed on admixture with authentic homoanisic acid.

Fraction 24 was a transition fraction. Fractions 25 to 28 contained sesquiterpenes. From 3 Gm. each of fractions 25, 27, 28, 0.6 Gm., 1.4 Gm., and 1.8 Gm. of hydrochloride, respectively, were obtained by the usual treatment with dry HCl and one recrystallization from glacial acetic acid. In each

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[1] Thanks are due to M. Louis Huguet, a forester with the Food and Agricultural Organization, working now in Mexico, for his assistance in procuring this sample of oleoresin.

case one or two recrystallizations brought the melting point to 58–59°, and the melting points were not depressed by admixture with the hydrochloride of *d*-longifolene.

PINUS HERRERAI

Pinus herrerai Martinez was discovered in 1939 in the State of Jalisco, Mexico, and described by Martinez (4) as a new species, independent from the closely related *P. teocote*. According to the older classification by Shaw (7), *P. herrerai* would be placed in the group *Australes*. Characterized by very rapid growth in its youth, this pine may reach a height of 100 feet at maturity. It possesses extremely soft and delicate needles, three to a bundle, and very small cones, usually 2 to 3 cm. long and only seldom reaching 4.5 cm. *Pinus herrerai* grows in the States of Jalisco, Sinaloa, Guerrero, Durango. It had never been reported before from Michoacán. The oleoresin sample for the present study was collected by Ing. Mário Avila González near Ciudad Hidalgo, Michoacán, at an altitude of 6,560 feet. The sample was accompanied, as always, with specimens of foliage and cones.

A batch of 4,805 Gm. of oleoresin was heated under reduced pressure so that at the end of the operation, temperature was 195° and vacuum was 1 mm. of mercury. Yield of turpentine amounted to 24.2%. The rosin was hard and brittle.

A batch of 1,012 Gm. of *P. herrerai* turpentine was fractionated with a 90-cm. long, 25-mm. inside-diameter Todd column packed with 3/32-inch single-turn glass helices. A reflux ratio of 10:1 was maintained.

After the head temperature reached 155°, fractions were taken approximately every 100 ml. until 81.6% of the charge had distilled. The properties of these fractions were the same, so the fractions were combined. The properties of fraction 3 were close to those of pure α-pinene. Accordingly, *dl*-α-nitrosochloride was prepared from fraction 3. Judging from a strong dextrorotation of this fraction, it apparently consisted largely of *d*-pinene, and the yield of *dl*-nitrosochloride was very poor. After recrystallization from chloroform by addition of cold methanol, 100 mg. of nitrosochloride was obtained. Its melting point, 103.5 to 104.5°, was not depressed when authentic *dl*-α-pinene nitrosochloride was admixed.

Fractions 4, 5, and 6 were mainly *d*- and *dl*-α-pinene. Fractions 7, 8, and 9 were intermediate fractions containing α-pinene.

Fraction 10 yielded no crystalline nitrosate. However, from 2.5 Gm. of fraction 12, 100 mg. of nitrosate m. p. 142–144°, was prepared. After recrystallization from chloroform by addition of methanol, the nitrosate melted at 146–147°, and its melting point was not depressed when authentic *d*-Δ^3-carene nitrosate was added.

A tetrabromide was prepared from 1 ml. of fraction 12. About 140 mg. was received, which after several recrystallizations from ethyl acetate, melted at 122.5–124°, its melting point was not depressed on admixture of authentic dipentene tetrabromide.

Fraction 17 contained an oxygenated compound. Attempted reactions with *p*-nitrobenzoyl chloride and 2, 4-dinitrophenylhydrazone, yielded no crystalline derivatives. Analysis of fraction 17: Found: C, 77.78%; H, 10.77%.

Fractions 19 and 20 are composed of sesquiterpenes. Fraction 20 was redistilled over sodium. A heart cut had the properties: b_{11}, 103–112°; d_4^{23}, 0.940; $n_D^{21.4}$, 1.4951; $[\alpha]_D^{23}$, +37.9°. Preparation of a hydrochloride was attempted using 3.5 Gm. of the heart cut. On recrystallization of the obtained product, 140 mg. of white crystals was received. They melted at 58.5–59°, and their melting point was not depressed by admixture of an authentic hydrochloride of *d*-longifolene.

PINUS PONDEROSA

Pinus ponderosa has an enormous range, from British Columbia to northern Mexico and from the Black Hills of South Dakota to the California coast. We have already reported in this series of articles on the composition of turpentine of *P. ponderosa* from the Sierra Nevada of California (2), from Arizona, Colorado, South Dakota, and northern Idaho (3), and from Utah (6). We have found that ponderosa pine turpentine throughout its range contains large amounts of Δ^3-carene; but at the same time, local races of this species can be characterized by presence or absence of certain minor ingredients of turpentine. For instance, myrcene is found only in the Pacific Coast forms of ponderosa pine and terpinolene only in the forms growing east of the Rocky Mountains.

The sample of oleoresin used in the present study came from the Santa Cruz mountains of California, just a few miles from the coast. This locality is separated from the pine-clad Sierra Nevada by a stretch of agricultural land about 100 miles wide. There is also an isolated stand of *P. ponderosa* at Mt. Hamilton, not far from Palo Alto, California. It was, therefore, interesting to determine the chemical composition of ponderosa pine turpentine from such an isolated and rather unusual locality.

Ten oleoresin samples from this locality were collected by Mr. Frank A. Hellenthal at his mountain ranch, near the town of Santa Cruz, California. These ten samples were distilled separately under reduced pressure (4 mm. of mercury), so that the temperature at the end of distillation was 180°. All ten samples of the thus obtained turpentine were laevo-rotatory, the rotation varied from −12.5 to −32.3°. Densities at 25° varied from 0.8532 to 0.8685 and indexes of refraction varied from 1.4732 to 1.4782.

A mixture of these samples had the following physical characteristics: d_4^{25}, 0.8549; n_D^{25}, 1.4749; $[\alpha]_D^{25}$, −23.2°.

Analyses of the turpentine indicate that *Pinus ponderosa* from the Santa Cruz coastal range possesses the same specific character as all previously investigated samples taken throughout the range of the species. This specific character is Δ^3-carene. *Pinus ponderosa* from Santa Cruz mountains also contains in its turpentine some β-myrcene, which we have found in all ponderosa pine samples from the Pacific coast. Rather unexpectedly, Santa Cruz ponderosa pine contained some *n*-undecane and possibly some *l*-β-phellandrene. These hydrocarbons are not found in more eastern samples of turpentines of this pine, but they are found (5) in turpentine of *Pinus coulteri*, which grows in the

coastal ranges of California, resembles *P. ponderosa*, and is the only other pine commonly attacked by the Western pine beetle (*Dendroctonus brevicomis* Lec.).

A charge of 324 Gm. of the *P. ponderosa* turpentine was distilled through a 90-cm. long 12-mm. inside-diameter Todd column, packed with 3/32-inch diameter glass helices. A 10:1 reflux ratio was maintained throughout. The results of the distillation are shown in Table I.

The refractive indexes and densities of the fractions were plotted with the refractive indexes as abscissas (8). The resulting graph indicated the presence of both α-pinene and β-pinene.

dl-α-Pinene nitrosochloride was prepared from fraction 2. The nitrosochloride, after recrystallization, melted at 103.5 to 104.5°, and its melting point was not depressed when authentic *dl*-α-pinene nitrosochloride was admixed.

Fraction 3 was a transition fraction containing both α- and β-pinene. Fractions 4 and 5 consisted mainly of *l*-β-pinene. An alkaline permanganate oxidation of 20 Gm. of fraction 5, yielded 2.7 Gm. of sodium nopinate. The sodium nopinate was converted into nopinic acid, which melted at 126.5° and showed no melting point depression on admixture with authentic nopinic acid. Fractions 6 and 7 were transition fractions containing mainly

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS HERRERAI, P. LAWSONI, AND P. PONDEROSA FROM SANTA CRUZ, CALIFORNIA

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate %	Density	Index of Refraction	Specific Rotation
	Pinus lawsoni (974 Gm. used)			d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
1	760	100–154	0.4	0.851	1.4611	...
2		154–155.5	25.8	0.853	1.4674	+41.6
3		155.5–157	11.5	0.852	1.4649	+39.9
4		157–159	10.7	0.853	1.4661	+35.5
5		159–161	2.4	0.854	1.4670	+31.5
6		161–163	2.0	0.854	1.4678	+28.0
7		163–165	2.4	0.854	1.4684	+24.2
8	760	165–166	1.5	0.855	1.4693	+21.3
9	14	51–55	5.3	0.856	1.4700	+19.7
10	14	56	1.5	0.858	1.4708	+14.8
11	14	56–59	20.7	0.859	1.4714	+14.3
12	14	59–60	2.0	0.856	1.4720	−2.7
13	14	60–62	0.6	0.850	1.4731	−21.0
14	14	62–67	0.8	0.849	1.4752	−30.0
15	14	67–69	0.3	0.848	1.4791	−24.8
16	14	69–71	0.5	0.852	1.4827	−14.3
17	14	71–72	0.6	0.854	1.4853	−6.6
18	14	72–74	0.9	0.856	1.4872	−2.6
19	14	74–80	0.3	0.861	1.4888	−2.6
20	14	80–92	0.4	0.885	1.4915	−4.5
21	14	92–94	0.2	0.918	1.4972	−5.0
22	14	94–98	0.7	0.942	1.5047	−3.4
23	14	98–103	0.4	0.952	1.5059	+5.3
24	14	103–114	0.5	0.945	1.5018	+17.0
25	14	114–118	0.6	0.939	1.4992	+33.2
26	14	118–121	0.8	0.937	1.5002	+42.4
27	14	121–122	2.5	0.934	1.5021	+47.5
28	14	122	1.1	0.929	1.5028	+47.1
Pot residue and loss			2.6			
	Pinus herrerai (1,012 Gm. used)			d_4^{24}	n_D^{24}	$[\alpha]_D^{25}$
1		120–154	0.4			
2	760	154–155	2.7	0.8535	1.4632	+49.1
3	760	155–155	78.5	0.8538	1.4640	+49.5
4	13	43–45	5.3	0.8545	1.4648	+49.5
5	13	45–47	0.6	0.8560	1.4668	+42.1
6	13	47–48	0.2	0.8581	1.4682	+36.2
7	13	48–50	0.2	0.8565	1.4695	+31.2
8	13	50–52	0.2	0.8559	1.4708	+25.2
9	13	52–54	0.1	0.8537	1.4718	+20.4
10	13	54–56	0.3	0.8514	1.4719	+18.1
11	13	56–58	0.2	0.8492	1.4719	+14.9
12	13	58–60	0.7	0.8464	1.4720	+16.5
13	13	60–64	0.3	0.8470	1.4731	+18.4
14	13	64–76	0.3	0.8534	1.4751	+12.2
15	13	76–83	0.3	0.8619	1.4753	+4.6
16	13	83–87	0.6	0.8754	1.4709	−7.1
17	13	87–89	0.4	0.8838	1.4708	−11.3
18	13	89–94	0.5	0.9082	1.4820	−0.7
19	13	94–99	0.3	0.9374	1.4912	+17.1
20	13	Above 99	1.2	0.9434	1.4950	+36.9
Pot residue and loss			6.7			

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate %	Density	Index of Refraction	Specific Rotation
	Pinus ponderosa from Santa Cruz (324 Gm. used)			d_4^{24}	n_D^{25}	$[\alpha]_D^{24}$
1	13	43–44	0.6	0.859	1.4696	−32.2
2		44–45	2.8	0.857	1.4692	−34.2
3		45–47	11.1	0.860	1.4730	−28.5
4		47–48	7.9	0.861	1.4741	−26.2
5		48–50	16.0	0.857	1.4751	−20.3
6		50–51	3.6	0.852	1.4743	−13.3
7		51–52	6.3	0.850	1.4727	−6.4
8		52–53	5.0	0.846	1.4720	−1.2
9		53–54	7.7	0.846	1.4711	+3.5
10		54–54.5	7.8	0.846	1.4704	+5.7
11		54.5–55	3.2	0.848	1.4704	+2.0
12		55–56	4.9	0.849	1.4705	−5.2
13		56–58	4.8	0.848	1.4714	−38.7
14		58–59	0.6	0.840	1.4721	−77.2
15		59–60	4.0	0.837	1.4722	−90.2
16		60–63	1.1	0.839	1.4733	−93.3
17		63–67	0.6	0.844	1.4778	−56.4
18		67–69	0.8	0.847	1.4809	−22.6
19		69–71	0.6	0.844	1.4783	−9.7
20		71–84	0.8	0.841	1.4691	−8.1
21		84–95	0.3	0.875	1.4761	−7.8
22		95–110	1.1	0.923	1.4891	−3.3
23	13	110–121	1.2	0.929	1.4971	−10.3
24			2.2	0.906	1.5038	−60.5
Pot residue and loss			5.0			

β-pinene. Fraction 8 had a low density which suggested the presence of β-myrcene, and a small amount of the maleic anhydride adduct of β-myrcene was prepared (3). The maleic anhydride adduct melted at 33° and showed no melting point depression on admixture with authentic maleic anhydride adduct of β-myrcene.

Fractions 9, 10, and 11 had positive rotations and the sweet odor characteristic of *d*-Δ^3-carene.

Five Gm. of fraction 10 was used to prepare 0.25 Gm. of a nitrosate (1). After recrystallization from chloroform by addition of cold methanol, the nitrosate melted at 145° and showed no melting point depression on admixture with authentic Δ^3-carene nitrosate.

Fractions 12 and 13 were transition fractions containing both *d*-Δ^3-carene and *l*-limonene. An attempt to prepare a nitrosite yielded a gummy precipitate indicating the possible presence of *l*-β-phellandrene.

The high negative optical rotations and the low densities of fractions 14 to 17 indicated the probable presence of *l*-limonene. Fraction 15 was redistilled over sodium and a heart cut with the following properties was received: d_4^{24}, 0.839; n_D^{23}, 1.4733. A tetrabromide was prepared from the redistilled fraction 15. After several recrystallizations from ethyl acetate, the tetrabromide which originally melted at 97–99° was separated into two parts. The most insoluble fraction melted at 121–123°, and the melting point was raised to 122–124° by admixture with dipentene tetrabromide. The other fraction melted at 103° and showed no depression in melting point on admixture with an authentic sample of the tetrabromide of *l*-limonene.

Fraction 20 showed a positive Schiff's test for aldehydes. No appreciable derivative was obtained with 2,4-dinitrophenylhydrazine reagent, however. The slight lowering of density and refractive index indicated the possibility of the presence of a small amount of undecane. By repeated shaking of fractions 19, 20, and 21 with concentrated sulfuric acid and finally with fuming sulfuric acid, until the acid was no longer darkened by the oil, a small amount of inert oil was obtained. The residual oil (0.4 Gm.) had the following properties: d_4^{24}, 0.7352; n_D^{24}, 1.4162; $[\alpha]_D$, 0.0: micro boiling point 195°; for $C_{11}H_{24}$.

Anal.—Calcd. for $C_{11}H_{24}$: C, 84.52; H, 15.48. Found: C, 84.27; H, 15.32.

Fractions 22, 23, and 24 are composed of sesquiterpenes. Attempted preparation of a hydrochloride yielded no crystalline derivative from fraction 23. Fraction 24 was redistilled over sodium at reduced pressure. The higher boiling fraction (4.1 Gm.) had the properties: d_4^{23}, 0.8921; n_D^{23}, 1.5061; $[\alpha]_D^{24}$, −71.1°. A hydrochloride (0.4 Gm.) was prepared from 3 ml of this fraction. The hydrochloride was recrystallized from glacial acetic acid twice; after this treatment it melted at 117–117.5° and showed no depression in melting point on admixture with authentic cadinene hydrochloride. The optical rotation of the hydrochloride was: $[\alpha]_D^{23}$, −40.8°; c = 0.98 in chloroform.

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Interaction Studies of Cationic Drugs with Anionic Polyelectrolytes I* Sodium Carboxymethylcellulose

By LLOYD KENNON† and TAKERU HIGUCHI

A study of the interaction of sodium carboxymethylcellulose with a number of cationic drugs indicated that even prior to the formation of visible reaction products, binding of the drug ions on the dissolved polyelectrolyte occurs. It has been shown that CMC forms complexes in this manner with the cationic drugs quinine, Benadryl®, procaine, and Pyribenzamine®. With amphetamine and ephedrine only the Donnan effect appears to be operative. Sodium chloride will to a great extent displace the bound cations from the CMC. A method utilizing a dialysis technique and Donnan equilibrium concepts has been presented.

THE INCORPORATION of polyelectrolytes in pharmaceutical systems is more prevalent presently than was formerly the case. The significance of their value in considerations of drug insolubilization, appearance, and stability, and also in prolongation of action is appreciated to a greater extent. The latter two facets of polyelectrolyte application are the ones which are apparently receiving the most attention in industrial pharmaceutical research currently.

Miller (1), in discussing timed release of medication from tablets and capsules, has drawn attention to the following fact. Prolongation of the time of therapeutic efficacy can be effected not only by lengthening the disintegration time of the dosage form, but also by administering a suitable insoluble form of the drug or a drug combination with ion-exchange resins or other adsorptive substances. Industrial application of this principle has been exemplified by the marketing of Foxalin, a digitoxin-CMC combination affording uniform sustained absorption without "digitoxic punch," and Stimalose, an amphetamine-caffeine-ionic bonding agent tablet combination which disintegrates rapidly but has a repository effect due to the extended release of the medication from its ionic bonding agent.

It is well known (2) that solutions of ampholytes such as proteins and amino acids exhibit properties which distinguish them sharply from ordinary nonelectrolytes. These characteristic properties are attributable to their unique dipolar or zwitter ionic structure. Thus, a dipolar ion has a very large dipole moment and is, in fact, a superpolar molecule surrounded by an intense electrostatic field. Hence, strong electrostatic interaction between such dipoles and real ions in solution is to be expected.

Higuchi and Lachman (3) have shown that the hydrolysis of benzocaine is inhibited when the latter is complexed with caffeine. The mechanisms involved in this example of the production of stability of an ester linkage in basic solution are sure to be capable of further extension and application. Use of certain ionic agents may make this easier, since the intermolecular binding forces involved will be greater than in the case cited.

The object of the present work was to determine the magnitude of the drug-binding property of a water-soluble polyelectrolyte and the effects of varying drug concentrations, the presence of neutral salt, and different cationic pharmaceuticals on this binding. The overall binding effect involved here may be considered to be the sum of that arising from the Donnan equilibrium and that produced by all other interactions. The latter types of binding are not amenable to complete and vigorous definition, but may be looked upon as being due to a combination of those forces which are significant in holding atoms together in such structures as crystals, molecules, and loose intermolecular complexes. The pharmaceuticals chosen were the hydrochloride salts of amphetamine, Benadryl®, procaine, Pyribenzamine®, ephedrine, and quinine.

Carboxymethylcellulose sodium was chosen as the polyelectrolyte for this initial study because it is familiar in pharmacy, being used as a thickener, a dispersing agent, and as a physical binder. The present communication is restricted to this agent only, although preliminary tests have shown it to be one of the weaker binders. The results of studies utilizing other polyelectrolytes will be reported in a future communication.

THEORY

Although these polyelectrolyte systems do not

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conform strictly to the classical Donnan systems, from a practical standpoint it is convenient to use these concepts in understanding the overall phenomena. Despite the fact that it is extremely difficult, for example, to establish ionic activity coefficients for these high molecular weight polyvalent ions, the effective degree of dissociation of the polyelectrolytes can be estimated by use of Donnan equations (7).

The equilibrium bearing his name was effectively defined by F. G. Donnan, in 1911, who investigated and developed the initial ideas of Wilhelm Ostwald, who, in 1890, noted that special effects must occur with ions in solution when at least one ionic species is constrained (4, 5, 6). It is not particularly stressed in texts that the Donnan equations developed are applicable only to systems in which the membrane divides the total volume into equal portions. When the two membrane-separated solutions are not of equal volume, as is the usual experimental case, the Donnan equations are somewhat more complex.

The system involved in this study is as follows: Diagram A represents the initial situation, B the system after equilibrium has been established.

$CMC^- \; c_1$	$D^+ \; c_2$	$CMC^- \; c_1$	$D^+ \; c_2 - \frac{y}{V_i}$
		$Na^+ \; c_1 - \frac{z}{V_i}$	
$Na^+ \; c_1$	$Cl^- \; c_2$	$Cl^- \; \frac{x}{V_i}$	$Cl^- \; c - \frac{x}{V_0}$
		$D^+ \; \frac{y}{V_i}$	$Na^+ \; \frac{z}{V_0}$
i	0	i	0

Diagram A: Initial State — Diagram B: Final State

Where: The i and 0 refer to the "inside" (polyelectrolyte side) and "outside" of the membrane, respectively; and:

x = MEQ of Cl^- inside
y = MEQ of D^+ inside
z = MEQ of Na^+ outside
c_2 = original concn. of D^+ and Cl^- outside
c_1 = original and final concn. of CMC^- inside
V_i = inside volume
V_0 = outside volume

Upon solving the Donnan equilibrium equations, we get:

$$x = \frac{(2ac_2 + c_1a^2) - \sqrt{(2ac_2 + c_1a^2)^2 + 4(a^2 - 1)(a^2c_2^2)}}{2(1 - a^2)}$$

Where: $a = \frac{V_0}{V_i}$ and concentrations are expressed as MEQ per unit volume, using V_i as the unit volume.

Hence, if the dissociation of the sodium salt of the polyelectrolyte can be determined so that c_1 of Diagrams A and B is known, a theoretical curve of drug cation concentration inside over drug cation concentration outside versus the amount of drug cation can be drawn. This curve then represents the situation when *only* Donnan-binding is existent. Comparison of this curve with one determined by experiment should then afford an insight into the interactions, if any, that are taking place between the polyelectrolyte anions and the drug cations.

The degree of dissociation of the polyelectrolyte can be determined also by use of Donnan concepts. The dissociated or the effective polyelectrolyte concentration is given by the expression (7):

$$[CMC^-] = \frac{(Cl^-)_0^2 - (Cl^-)_i^2}{(Cl^-)_i}$$

Where:

$(Cl^-)_0$ = concn. of chloride on outside
$(Cl^-)_i$ = concn. of chloride on inside

in a system in which the sodium salt of the polyelectrolyte is brought into equilibrium with sodium chloride across a semipermeable membrane. The slope of the line when the effective polyelectrolyte concentration is plotted against total polyelectrolyte added gives the degree of dissociation.

The above-mentioned theoretical curve of C_i/C_0 versus amount of drug is shown in Fig. 2–4. Deviations of the experimentally determined values from this theoretical curve then indicate that the Donnan-binding is not the only effect taking place within these systems.

EXPERIMENTAL

Gross Observations.—The method employed consisted of simple mixing of solutions of the drug and CMC. A 5-cc. portion of 0.075 *M* drug solution was poured into 5 cc. of 1% CMC solution.

Equilibrium Studies.—Solutions of the drugs studied were brought into equilibrium with CMC across a semipermeable membrane both in the presence and absence of sodium chloride. The membrane bags were fashioned from Visking cellulose casings. The systems were shaken packed in ice until equilibrium was established. The attainment of equilibrium was ascertained by noting that $C_0 = C_i$ in blank runs, that is, in runs with no polyelectrolyte present.

Drug concentrations were adjusted so that, on an equivalent basis, they would equal 10%, 15%, 25%, 40%, 60%, and 100% of the amount of CMC. The total system at each point contained one MEQ of CMC, with the varying amounts of drug: 0.1, 0.15, 0.25, 0.40, 0.60, and 1.00 MEQ. The volume of the bags was 30 cc.; the total volume of the systems was 130 cc.

The dissociation of the sodium salt of the polymer was determined similarly by bringing the polymer salt into equilibrium with sodium chloride across the cellulose membrane. Chloride ion assays (8) were accomplished by a titration procedure utilizing 0.01 *N* silver nitrate with fluorescein as an adsorption indicator. The method has been described previously (7, 9).

Reagents.—Sodium carboxymethylcellulose (CMC-70, Low Viscosity, Hercules Powder Company). Ephedrine hydrochloride, U. S. P. crystalline (Mallinckrodt Chemical Works). *dl*-Amphetamine hydrochloride powder (Herman Meyer Drug Company). Pyribenzamine® hydrochloride powder (Ciba Pharmaceutical Products, Incorporated). Benadryl® hydrochloride powder (Lot #539069, Parke, Davis and Company). Quinine hydrochloride, U. S. P. (Merck and Company,

Incorporated). Procaine hydrochloride, U. S. P. crystalline (Merck and Company, Incorporated).

Method of Drug Analysis.—A model DU Beckman spectrophotometer was used in the analysis of the internal and external solutions, properly diluted, of the following substances at the wave length values listed: procaine hydrochloride, 290 mμ; ephedrine hydrochloride, 257 mμ; amphetamine hydrochloride, 257 mμ; Benadryl® hydrochloride, 258 mμ; Pyribenzamine® hydrochloride, 306 mμ; quinine hydrochloride, 331 mμ.

RESULTS AND DISCUSSION

Gross Observations.—When solutions of the drugs were mixed with CMC solution, no visible turbidity or precipitation was observed with the hydrochloride salts of ephedrine, procaine, amphetamine, and Pyribenzamine®; with Benadryl® hydrochloride a very slightly turbid, colloidal solution resulted; with quinine hydrochloride a stringy, sticky precipitate was formed. The precipitates appear to be caused by the exceeding of the solubility of the complex formed between the polyelectrolyte anion and drug cation. It seems, however, that not all drug-CMC interaction is manifested by the formation of visible aggregates.

Equilibrium Studies.—As described under "Experimental," further studies were undertaken to determine the formation of drug-CMC complexes. Figure 1 indicates that sodium carboxymethylcellulose is 60% dissociated in solution. This value agrees with that found for a different batch in a slightly different manner (7). From this information, the expected Donnan effects were determined. It has been assumed that this value for the dissociation or "anionic activity" holds throughout the dilute range studied. The straight-line relationship plotted in Fig. 1 and the self-consistency of the data seem to bear out this assumption.

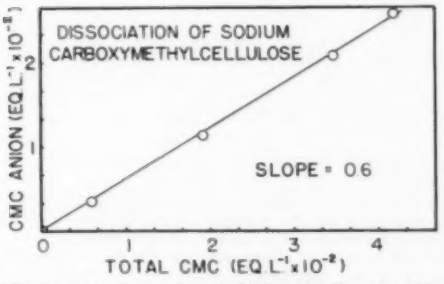

Fig. 1.—Plot shows that CMC is 60% dissociated in solution.

The ratios of the inside drug concentration to outside drug concentration, at constant CMC, are plotted against the total amount of drug present in Fig. 2–4. Figure 2 shows that ephedrine and amphetamine behave substantially as predicted by the Donnan theory. Thus, with the exception of the slight deviation noted toward the more concentrated end of the range, it appears that CMC does not bind amphetamine or ephedrine cations. Figure 3 illustrates that Pyribenzamine® and pro-

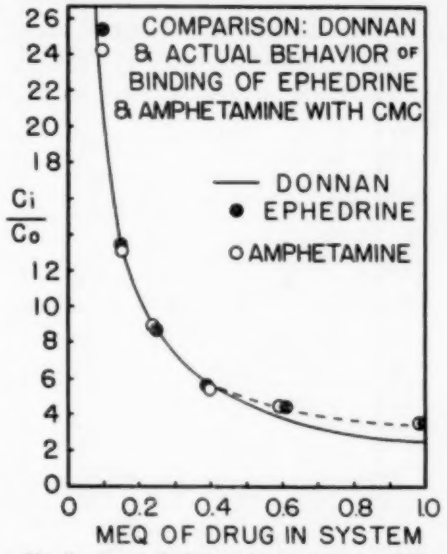

Fig. 2.—Graph indicates that non-Donnan binding of ephedrine and amphetamine by sodium carboxymethylcellulose is absent. The ratio of the concentration of the drug inside to outside of the dialysis bags, at equilibrium, has been plotted against the amount of drug present.

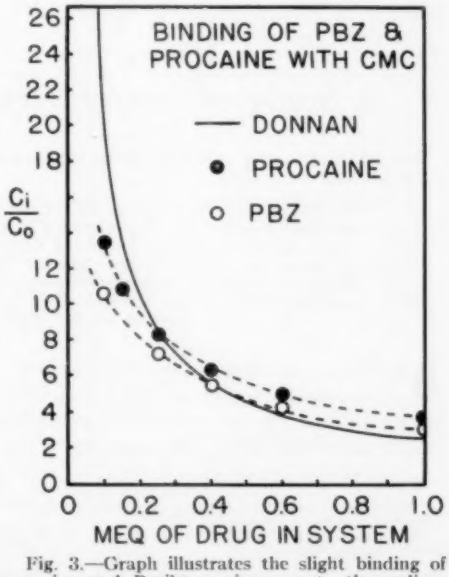

Fig. 3.—Graph illustrates the slight binding of procaine and Pyribenzamine® on to the sodium carboxymethylcellulose.

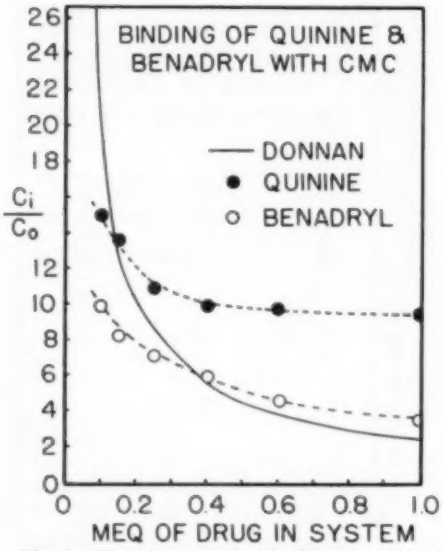

Fig. 4.—Plot demonstrates the binding effect of sodium carboxymethylcellulose on quinine and on Benadryl®.

caine show marked deviations from Donnan-only behavior. Hence, it appears that some cations are bound such that the effective polyelectrolyte anion concentration is reduced and the system then follows a different theoretical curve. Figure 4 demonstrates the greater deviation which occurs with Benadryl® and quinine with CMC; it seems reasonable to interpret this as evidence of a greater tendency for CMC to bind Benadryl® and quinine. As noted previously, visible aggregates also resulted in this latter case.

Because experimentally determined and theoretical curves intersect, it is essential that in a study such as this, a range of concentrations be employed so that some possible false conclusions are avoided.

In the presence of isotonic sodium chloride, which also eliminates the significance of the Donnan effect, the drug C_i/C_o ratios drop approximately to 1. The values for quinine lie between 1.25 and 1.50. The relative excess of sodium ions have largely replaced the drug ions from their positions on the CMC. This ability of the sodium ions to replace the drug cations from the CMC is readily understandable when the great differences in the ionic radii concerned are considered. Not only would the sodium ion have the ability to achieve a closer point of approach by virtue of its size, but also its charge density would be more intense than that of the relatively large drug cations. Thus the sodium ions have characteristics which would make them the preferred gegen ions for the CMC. This study then actually provides a method for determining whether the polyelectrolyte has any affinity for the drug cations when the sodium ions are not in excess.

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A Study of Cocoa Syrups for Taste Preference*

By ARTHUR W. REID and CHARLES H. BECKER

Eleven different formulas of cocoa syrups, including the official one, were evaluated statistically for taste preference by an untrained panel of thirty subjects. Some cocoa syrups contained an added flavor such as salt, imitation butterscotch, and imitation rum concentrates. Others were prepared by the cold process instead of the hot, and some syrups were prepared using Dutch process cocoa. These were compared with Cacao Syrup, U. S. P. The official syrup, alone or with the addition of cocoa butter, was preferred for taste.

BECAUSE COCOA SYRUP is a popular vehicle and an excellent disguiser of bitter and salty tasting drugs, it was the purpose of this study to modify the official cocoa syrup to see which formula was most acceptable for taste. This was done by adding a flavor such as salt and cocoa butter to some syrups, using Dutched cocoa in some formulas and making other syrups by the cold process instead of the hot. The syrups were evaluated statistically for taste preference by an untrained panel of thirty subjects. In a later paper the disguisability effectiveness of these syrups against quinine hydrochloride will be given.

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EXPERIMENTAL

The majority of the chemicals employed in the preparation of the imitation flavor concentrates used were those generally employed by flavor manufacturers. They were of food grade. All others were of U. S. P. or N. F. quality. The manufacturers of the chemicals that were not official are indicated in the formulas by the following abbreviations in parentheses following the chemicals: Florasynth Laboratories, Inc. (F1); Fritzsche Brothers, Inc. (Fr); Synfluer Laboratories Inc. (S); S. B. Penick and Co. (P); George Lueders and Co. (L); Merck and Co., Inc. (M); and Magnus, Mabee and Reynard Inc. (MR).

When extremely small quantities of the chemicals were called for, 10% or 1% tinctures were made, and aliquot portions taken to insure accurate measurements. The concentrations were made by simple solution, as all chemicals were soluble in the solvents used.

The formulas for the imitation flavor concentrates used appear below:

Imitation Butterscotch Concentrate

Vanillin	1.5	Gm.
Extract of Foenugreek (P)	1.35	Gm.
Butyric Acid (P)	0.25	ml.
Caproic Acid (S)	0.25	ml.
Diacetyl (S)	1	ml.
Ethyl Myristate (F1)	1	ml.
Oil of Lovage	0.0015	ml.
Oil of Tar, Rectified	0.006	ml.
Orange Oil	0.5	ml.
Ginger Oil	0.25	ml.
Propylene Glycol, a sufficient quantity		
To make	100	ml.

Imitation Rum Concentrate

Ethyl Oxide Hydrate (L)	60	ml.
Butyric Ether (F1)	20	ml.
Ethyl Acetate (M)	20	ml.
Oil of Lemon	0.20	ml.
Oil of Cinnamon	0.20	ml.
Oil of Neroli	0.08	ml.
Balsam of Peru	0.16	ml.
To make about	100	ml.

The thirteen syrups used in this investigation were prepared in the laboratory and designated T_1, T_2, etc. They are described in Table I. The cocoa, supplied by the Hershey Chocolate Corporation, was of two types: American process, with a fat content of $15^1/_2$–$16^1/_2$ per cent, and Dutch process with a fat content of $17^1/_2$–18 per cent. All cocoa syrups were made according to the official formula, with the exception of T_7, in which skimmed milk was substituted for distilled water. All cocoa syrups, with the exception of T_3 and T_4, were prepared by the method official for cacao syrup. Syrups T_3 and T_4 were prepared by a method hereafter designated as the "cold" method in which cocoa and sugar-gelatin solution were cooled before mixing. An electric mixer was used to facilitate dispersion and insure smoothness of the cocoa syrups. All syrups were preserved against mold growth by the addition, in 1:3,000 concentration, of an equal mixture of methyl, propyl, and butyl *p*-hydroxybenzoates.

A nonselected panel of thirty adult students, males and females in good health, was used for taste preference tests. The general nature of the problem was made clear to them. Tasting was conducted in pairs with each taster being required to select (rank) the preferred sample, as the only decision he could make. Tasters received 4 ml. samples of the syrups in small paper cups and were instructed to use water to rinse their mouths between tests.

Series 1.—First objectives were limited to study the effect of cocoa preparation on preference, and only syrups T_1, T_2, T_3, and T_4 were used. These were given in pairs to the thirty subjects or judges in a balanced design, such that any two syrups were given together once and once only. This design required six pairs of syrups to be compared. To avoid taste fatigue, the subjects were divided into two groups, and tasting was completed at two sessions. At the first session, one group tasted three pairs selected at random, and the other group tasted the remaining three pairs. At the second session, each group tasted the three pairs not previously compared.

If it is assumed that no preferences existed among the subjects with respect to the four syrups, we would expect the preferences for the two syrups of of any one pair to be approximately equally divided. If the departure from the expected equal division of preferences is "large," the result is said to be statistically significant, and the hypothesis of no preference is rejected. Working at a prescribed level of probability, say the five per cent level, such a large or larger departures would on the average occur

TABLE I.—COCOA SYRUPS USED IN TASTE PREFERENCE TESTS

Syrup	Official Title	Type of Cocoa (Process)	Method of Preparation	Flavor Added
T_1	Cacao Syrup U. S. P.	American	Hot	None
T_2		Dutch	Hot	None
T_3		American	Cold	None
T_4		Dutch	Cold	None
T_5		American	Hot	0.1% Imitation butterscotch
T_6		American	Hot	0.1% Peppermint oil
T_7		American	Hot	Skimmed milk for distilled water
T_8		American	Hot	1% Cocoa butter
T_9		American	Hot	0.25% Salt
T_{10}		American	Hot	0.1% Imitation rum
T_{11}	Syrup U. S. P.	None	Hot	None
T_{12}		Dutch	Hot	0.25% Salt
T_{13}		Dutch	Hot	0.1% Imitation butterscotch

TABLE II.—RESULTS OF TASTE PREFERENCE TESTS, SERIES 1

Pairs	Preference for Former	Preference for Latter	Significance of Preferences
T_1, T_2	23	7	Significant at 1%
T_1, T_3	19	11	Not significant
T_1, T_4	18	12	Not significant
T_2, T_3	15	15	Not significant
T_2, T_4	13	17	Not significant
T_3, T_4	9	21	Significant at 5%

TABLE III.—TOTAL NUMBER OF FIRST PREFERENCES, SERIES 1

Method of Preparation	Type of Cocoa American	Type of Cocoa Dutch
Hot	60 (T_1)	35 (T_2)
Cold	35 (T_3)	50 (T_4)

TABLE IV.—CHI SQUARE TESTS ON DIFFERENCES AMONG THE FOUR SYRUPS REGARDED AS A 2 × 2 FACTORIAL SYSTEM

Sources of Variation	χ^2 Value	Degree of Freedom	Significance
American vs. Dutch Cocoa ($T_1 + T_3$) vs. ($T_2 + T_4$)	0.56	1	Not significant
Hot vs. Cold Method of Preparation ($T_1 + T_2$) vs. ($T_3 + T_4$)	0.56	1	Not significant
Interaction ($T_1 + T_4$) vs. ($T_2 + T_3$)	8.89	1	Significant at 1%
Total	10.00	3	Significant at 5%

by chance only once in twenty experiments.

With thirty subjects or judges, the normal curve approximation (calculated from

$$\frac{(x - 15) - 1/2}{\sqrt{30.1/2.1/2}} =$$

1.96 and solving for x. The half in the numerator is the so-called "correction for continuity") to the binomial distribution requires at least twenty-one judges to prefer a particular syrup for that preference to be significant at the five per cent level. The corresponding figure for significance at the one per cent level is twenty-three.

Table II gives the preferences obtained together with their significances. The four syrups compared were not entirely distinct from one another. They formed what is known as a 2 × 2 factorial system (two types of cocoa and two methods of preparation). The significances of each of these two factors and their interaction were tested after first pooling the preferences (Table III) of Table II.

Table IV shows that the assumption of no preferences among the subjects with respect to the four syrups must be rejected (with 95% confidence). The table also shows that there was no *average* difference between either the two types of cocoa or the two methods of preparation. The highly significant interaction between types of cocoa and methods of preparation means that the difference between the two methods of preparation for American type cocoa was different from that for the Dutch type; or, equivalently the difference between the two types of cocoa depended on whether the syrups were prepared by the "hot" or the "cold" method. The recommendation is to use the "hot" method for the American cocoa and the "cold" method for the Dutch cocoa.

TABLE V.—RESULTS OF TASTE PREFERENCE TESTS, SERIES 2

Pairs	Preference for Former	Preference for Latter	Significance of Preferences
T_1, T_5	19	11	Not significant
T_1, T_6	19	11	Not significant
T_1, T_7	17	13	Not significant
T_1, T_8	11	19	Not significant
T_1, T_9	16	14	Not significant
T_1, T_{10}	17	13	Not significant
T_1 vs. Others	99	81	Not significant

TABLE VI.—RESULTS OF TASTE PREFERENCE TESTS, SERIES 3

Pairs	Preference for Former	Preference for Latter	Significance of Preferences
T_1, T_8	14	16	Not significant
T_1, T_{12}	23	7	Significant at 1%
T_1, T_{13}	20	10	Not significant
T_8, T_{12}	18	12	Not significant
T_8, T_{13}	21	9	Significant at 5%
T_{12}, T_{13}	18	12	Not significant
($T_1 + T_8$) vs. ($T_{12} + T_{13}$)	82	38	Significant at 1%

Series 2.—In this series, T_1 was compared in turn with each of T_5, T_6, T_7, T_8, T_9, and T_{10}. Table V shows that the overall comparisons between T_1 and the other syrups were not significant, so that the addition of flavors did not make any significant difference one way or the other.

Series 3.—In this series, syrup T_1 was compared with syrups T_8, T_{12}, and T_{13}. Table VI shows that at the one per cent level of significance, syrup T_1 was preferred to syrup T_{12} and at the five per cent level, syrup T_8 was preferred to T_{13}. The table also shows that syrups T_1 and T_8 prepared with American cocoa were significantly preferred to syrups T_{12} and T_{13} prepared from Dutch process cocoa.

The Effect of Certain Non-narcotic Analgetics on Diuresis and Liver Sulfhydryl Concentration in the Albino Rat*

By D. J. COOPER†, T. S. MIYA, and L. D. EDWARDS‡

It was found that bilateral and unilateral hind-leg rubber band tourniquets produced an initial antidiuretic action when applied for two hours to hydrated rats. A much greater effect was produced upon release of the tourniquets. A comparable response was exhibited by formalin injection bilaterally into the hind-leg ankle joints. This antidiuretic response to pain was alleviated by certain non-narcotic analgetics. The toxicity of certain non-narcotic analgetics employed in this investigation appeared to be greatly increased by hydration of the animals. It was found that hind-leg ischemic pain produced a significant diminution in the total nonprotein sulfhydryl (TNPSH) concentration of the liver. This was prevented by treatment with acetylsalicylic acid.

IT HAS BEEN DEMONSTRATED that sustained pain may lead to various physiological changes in animals (1, 2). Ischemic pain more nearly approximates the deep pain associated with disease. This investigation was made to (*a*) attempt to devise a method of evaluating the non-narcotic analgetics in laboratory animals employing a physiological function as an index of measurement; (*b*) determine the relative efficacy of certain non-narcotic analgetics against ischemic pain, and (*c*) to obtain additional information as to possible mode of action of these drugs.

EXPERIMENTAL

The Effect of Non-Narcotic Analgetics on Diuresis in the Hydrated Rat Subjected to Pain.—A decrease in urine excretion occurs in rats exposed to varied stimuli as electrical stimulation of the feet, strange environment and noise (3), exercise (4), surgical trauma (5), flashing lights (6), histamine injection (5), (3), epinephrine (4), hind-leg tourniquets (7), and formaldehyde (8).

An attempt to relieve the antidiuretic response to noxious stimuli by non-narcotic analgetics was made in this phase.

The method used was a modification of that described by Birnie, *et al.* (9). Adult albino rats of both sexes, weighing approximately 200 Gm. and fed on Purina Laboratory Chow, were used. The rats were fasted for 12–16 hours, but allowed water *ad libitum*. They were placed in metabolism cages in groups of four. The cages were divided into four compartments to minimize excitement and to prevent disturbance of one rat by another. Urine was collected in graduated cylinders. Each rat was given two doses of tepid water, two hours apart, by stomach tube. Each dose corresponded to 5% of its body weight (5 cc. per 100 Gm. of body weight). Just prior to the administration of the second dose of water the volume of urine excreted in the previous two hours was recorded (V_2). The animals were prodded gently to stimulate emptying of the bladder before each measurement. Beginning with the time of the last gavage, the volume of urine was recorded at thirty-minute intervals for a period of two hours until the peak of diuresis had been observed. The formula used to determine urine excretion was as follows:

$$\text{Percentage urine excretion} = \frac{V_t}{2V_1 - V_2} \times \frac{100}{1}, \text{ where}$$

V_1 = the volume of water administered in each dose,
V_2 = the volume of urine excreted in the two hours before the second gavage, and
V_t = the volume of urine excreted during the period "t" minutes after injection (t = 30, 60, 90, 120 min.).

The animals were used for succeeding experiments after not less than a ten-day interval.

Pain Stimulus.—Ischemic pain was produced by bilateral or unilateral rubber band tourniquets (10), applied and removed at the time of the first and last gavages, respectively. The rubber bands were tied securely just above the ankle joint to occlude all blood vessels. An animal holder was employed so that etherization and excess excitement was avoided. In the case of formaldehyde-induced pain, a 2.5% solution was injected bilaterally into the ankle joints of the test animals immediately after the last gavage.

Drug Administration.—The test substance was administered in the last gavage. All drugs were suspended in 1% sodium alginate. Control animals received an equal volume of sodium alginate. In instances where a divided dose was given, the animal received the second portion thirty minutes after the last gavage in 2.5% of its body weight of tepid water.

RESULTS

Bilateral hind-leg tourniquets produced an initial

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antidiuretic action when applied to hydrated rats and a much greater effect upon removal. A comparable response was exhibited by unilateral hind-leg tourniquets and by formalin injection. In the latter case peak antidiuretic activity occurred thirty minutes after the injections. From the results obtained in these experiments, it was observed that bilateral tourniquets offered no advantage over unilateral tourniquets (Fig. 1). Thus, the latter were employed for this investigation. Alternate hind-legs were used for two determinations with tourniquets and the animals were also used for two determinations as controls.

Acetylsalicylic acid, 300 mg./Kg., administered to rats with ischemic pain produced an increase in urine excretion thirty minutes after administration ($P < 0.02$). There was no significant difference in diuresis after this thirty-minute period. This suggested that the drug action occurred during this period. Divided doses of the drug were administered for verification. There was a significant increase in urine output during the thirty-minute period immediately following both doses (300 mg./Kg. per dose) at $P = .01$. However, a second dose of 150 mg./Kg. after an initial 300 mg./Kg. had no effect (Fig. 2).

Other drugs in which there was a significant difference in treated animals with tourniquets as compared to control animals with pain at the thirty-minute intervals after administration are listed with their respective dosages in Table I.

Aminopyrine, 450 mg./Kg., which is tolerated by normal rats produced approximately 50% mortality in hydrated animals. It is possible that hydration of the animals greatly increases the toxicity of certain drugs.

The comparison between the average of tourniquet-control urine excretion and the averages of all groups simultaneously treated with drugs and tourniquets reveal that there is a significant difference at the 5% level for the thirty-minute interval. The Duncan test (11) shows that sodium salicylate, 150 mg./Kg. and Bufferin®, 450 mg./Kg. (dose calculated as acetylsalicylic acid) were more effective than any other treatment at the thirty-minute interval (Table I). Nonorthogonal comparisons (12) show that sodium salicylate, sodium acetylsalicylate, and Bufferin® exert a significant effect at the 5% level for the 60-minute (cumulative) interval. All other groups failed to do so at this interval.

There was no significant difference in urine excretion rate between animals subjected to pain and receiving drug treatment when compared to normal animals without tourniquet or normal animals receiving the respective drugs. We can conclude that drug treatment of normal animals had no effect on diuresis, but it antagonized the antidiuresis induced by a noxious stimulus.

Formalin injected animals treated with acetylsalicylic acid showed results comparable to those animals with tourniquet-induced pain.

Determination of Antidiuretic Substance in the Blood Serum.—This study was made to clarify the mechanism by which certain agents, particularly acetylsalicylic acid, inhibit the antidiuretic response to noxious stimuli.

Ginsburg and Heller (13) reported that normal rat serum contains two antidiuretic principles, a labile substance which disappears on standing and a stable

TABLE I.—SUMMARY OF THE EFFECT OF NONNARCOTIC ANALGETICS ON THE DIURESIS OF HYDRATED RATS SUBJECTED TO HIND-LEG ISCHEMIA

Treatment (Drug plus Tourniquet)	No. of Groups[a]	Noncumulative % Urine Excretion (Min. after Drug Administration) 30	60
Controls, tourniquet	15	5.5	17.5
Controls, no tourniquet	10	14.6	18.6
Acetylsalicylic acid, 300 mg./Kg.	6	10.8[b]	10.2
Salicylamide, 150 mg./Kg.	5	13.2	17.6
Antipyrine, 75 mg./Kg.	10	10.2	17.2
Aminopyrine, 100 mg./Kg.	6	9.6	13.2
Sodium salicylate, 150 mg./Kg.	5	30.1	32.3
Sodium acetylsalicylate 150 mg./Kg.[c]	5	17.3	24.6
Bufferin®, 450 mg./Kg.[c]	6	24.5	28.0
Acetylsalicylic acid, 300 mg./Kg. plus PABA 300 mg./Kg.	6	8.9	17.3
Acetophenetidin, 150 mg./Kg.	5	10.7	17.9
Acetylsalicylic acid, 300 mg./Kg. plus Formalin injection	4	16.3	23.9
Controls, formalin	4	2.3	24.3

[a] Four rats per group.
[b] For all drugs, urine excretion was significantly higher than tourniquet controls at the 30-minute interval.
Underscore: Significantly higher urine excretion than any other treatments, after Duncan (11).
Double underscore: Significantly higher at this interval, all other drugs produced no significant difference in urine excretion rate at 60 minutes when compared to tourniquet controls.
[c] Calculated as acetylsalicylic acid.

TABLE II.—SUMMARY OF THE EFFECT OF ISCHEMIC PAIN ON THE ANTIDIURETIC SUBSTANCE IN THE SERUM OF RATS

Treatment (Donor Animals)	Serum Injected (cc.)	No. of Rats	Diuretic Response Test Animal (cc.)[a]
Fresh serum			
Controls	0.4	7	0.17 ± 0.14[c]
Tourniquets[b]	0.4	7	0.99 ± 0.33
Stable ADS			
Controls	0.4	7	0.29 ± 0.11
ASA[d]	0.4	7	0.30 ± 0.08
Stable ADS			
Control	0.2	8	0.48 ± 0.06
	0.4	8	0.76 ± 0.08
Tourniquets	0.2	8	0.87 ± 0.13
	0.4	8	1.18 ± 0.30
Stable ADS			
Tourniquet	0.2	8	0.73 ± 0.07
	0.4	8	1.0 ± 0.03
Tourniquet with ASA[e]	0.2	8	0.33 ± 0.01
	0.4	8	0.53 ± 0.07

[a] Diuretic response calculated as: cc. secreted 15 minutes prior to serum injection minus cc. secreted 15 minutes subsequent to injection.
[b] Significantly different from controls ($P < 0.01$).
[c] Standard error of mean.
[d] Not significantly different from controls.
[e] ASA—Acetylsalicylic Acid, 600 mg./Kg. divided dose.

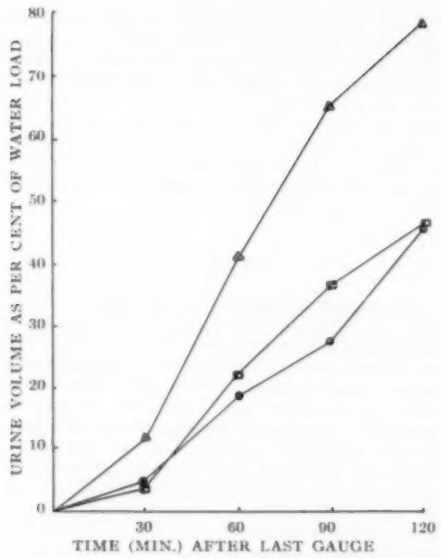

Fig. 1.—The effect of pain on diuresis in the hydrated rat. Legend: Control △——△, unilateral tourniquet □——□, bilateral tourniquet ○——○

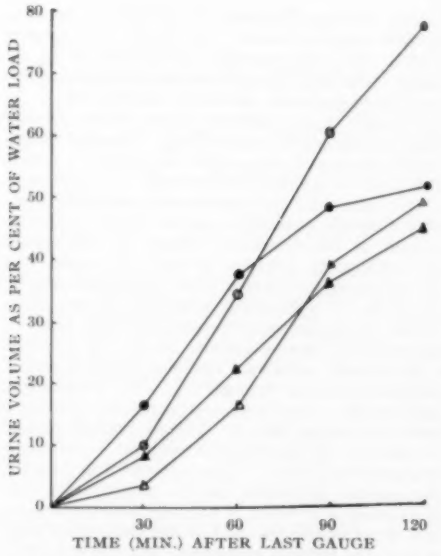

Fig. 2.—The effect of acetylsalicylic acid (ASA), 600 mg./Kg. and 450 mg./Kg., divided dose, on diuresis in the hydrated rat. Legend:

	No Tourniquet	Tourniquet
Control	○——○	△——△
ASA, 600 mg./Kg. (300 + 300)		●——●
ASA, 450 mg./Kg. (300+ 150)		▲——▲

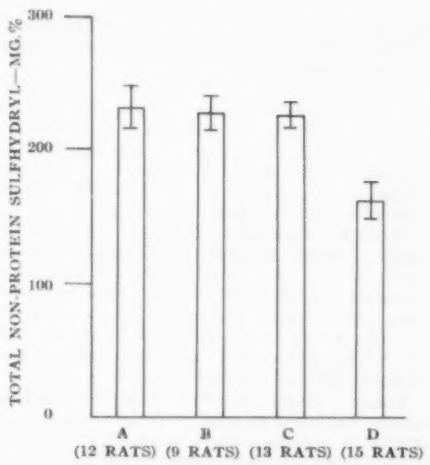

Fig. 3.—The effect of acetylsalicylic acid and/or bilateral hind-leg tourniquets on the sulfhydryl content of the liver of hydrated rats. Legend: A. Controls—no Tourniquets (231.3 mg.% ± 15.2). B. Acetylsalicylic acid 300 mg./Kg. at the beginning of the experiment and the same dose repeated 4 hours later (228.8 mg.% ± 12.2). C. Same treatment as B, but tourniquets applied for 4 hours (226.9 mg.% ± 9.5). D. Tourniquets applied for 4 hours (157.7 mg.% ± 11.4). All animals sacrificed 30 minutes after removal of tourniquets and/or administration of last dose of acetylsalicylic acid.

antidiuretic substance (ADS) which remains for many hours. A noxious stimulus produces an increase in the antidiuretic titer of the blood (14). The possibility exists that the non-narcotic analgetics tested in this investigation relieve the pain-induced antidiuresis by some mechanism which decreases the amount of circulating antidiuretic substance. Assays for the "stable" and "unstable" ADS in rat serum were conducted by a modification of the intravenous method of Jeffers, *et al.* (15).

To determine the antidiuretic response (R), the urine secreted by the test animal in the 15 minutes subsequent to injection was subtracted from that for the same period just prior to injection. A four-point assay was employed to determine relative potency. Randomized doses of 0.2 cc. and 0.4 cc. of serum of not more than two different serums was the limit for a single animal. A constant rate of diuresis was assured before injections were made.

Donor Animals.—Male albino rats, weighing 250–300 Gm., were fasted for 16–18 hours prior to the experiment, but allowed water *ad libitum*. The animals were hydrated by two gavages of tepid water, 5% of their body weight, with a two-hour interval between gavages. Tourniquets were applied and removed at the time of the first and last gavages, respectively. Treated animals received acetylsalicylic acid, 600 mg./Kg., divided dose. The first portion of 300 mg./Kg. was administered in the last gavage, and the last portion was given thirty minutes later. All animals were killed within thirty minutes after

the last administration of the drug. The animals were anesthetized by alcohol and blood was obtained by cardiac puncture.

Assays.—(*a*) *Assay of Fresh Serum.*—The blood was quickly centrifuged and 0.4 cc. of serum was immediately injected intravenously into the prepared rat (15). The injection was made within ten minutes after obtaining the blood. Assays were conducted on serum from control animals and animals with unilateral hind-leg tourniquets.

(*b*) *Stable Antidiuretic Substance.*—Acetone extracts of stable antidiuretic substance from the serum of rats were prepared by the method of Erspamer and Sala (16). Assays were performed using serum from animals receiving acetylsalicylic acid vs. controls; animals subjected to ischemic pain vs. controls; and animals subjected to ischemic pain with and without acetylsalicylic acid.

Results of Assays.—(*a*) *Fresh Serum.*—Serum from animals subjected to pain produced a marked inhibition in diuresis. There was a slight change in diuresis when the serum from normal animals was administered intravenously. The difference between serum from animals with pain and control animals was highly significant ($t = 9.1$).

(*b*) *Stable Antidiuretic Substance.*—There was no significant difference between the serum from control animals with tourniquets and serum from animals receiving acetylsalicylic acid, 600 mg./Kg., divided dose ($t = 0.6$).

The fresh serum from control animals was not significantly different from the acetone extracts of serum from control animals ($t = 1.95$).

A four-point design of statistical analysis was employed as described by Burn (17). According to this test, one cc. of serum from animals without tourniquets is estimated to contain the antidiuretic activity of 0.39 cc. (Fiducial limits 0.302–0.504) of serum from animals with tourniquets and one cc. of serum from animals with ischemic pain and treated with acetylsalicylic acid, 600 mg./Kg., was estimated to contain the antidiuretic activity of 0.28 cc. (Fiducial limits 0.069–0.469) of serum from animals with ischemic pain but no treatment.

Amperometric Titration for Total Nonprotein Sulfhydryl Content of the Liver of Hydrated Rats.—A quantitative determination and correlation of certain biochemical changes induced by noxious stimuli and the modification of these changes by non-narcotic analgetics may afford a means of evaluating such agents.

A pronounced diminution in the total nonprotein sulfhydryl content of the liver occurs on injecting adrenalin in rats (18), exposing mice to cold (19), or to cold and restraint (20), or restraint alone (21). The changes which occur in total nonprotein sulfhydryl concentration are presumably due primarily to changes in glutathione (18, 19). Experiments were conducted to investigate the changes which might occur in liver sulfhydryl content when hind-leg tourniquets were applied to rats and to observe the action of acetylsalicylic acid upon this change.

The apparatus used was essentially the same as that described by Kolthoff and Harris (22). The amperometric titration of sulfhydryl groups was performed using a rotating platinum indicator electrode and a mercury-mercuric iodide reference electrode. The animals were treated with 600 mg./Kg. of acetylsalicylic acid as already described under "Donor Animals" for the study on antidiuresis except that four hours elapsed between gavages. Tourniquets remained on for four hours. Thirty minutes after the second dose of the drug the animals were sacrified by a blow on the head and portions of the liver (300–400 mg.) were immediately excised and frozen with dry ice. After weighing the liver pieces, the nonprotein sulfhydryl concentration was determined according to the method of Benesch and Benesch (23).

RESULTS

The method of Duncan (11) was used to determine the significant differences between the means of the following at the 5% level: (*a*) control animals vs. animals with ischemic pain, (*b*) animals with ischemic pain vs. animals with ischemic pain treated with acetylsalicylic acid. Hind-leg tourniquets produced a pronounced lowering of the total nonprotein sulfhydryl concentration in the livers, which was protected by administering acetylsalicylic acid. There was no significant difference in the total sulfhydryl concentration of control animals, animals treated with acetylsalicylic acid without tourniquets, and acetylsalicylic acid with tourniquets (Fig. 3). More extensive investigation of these observations are now being conducted in this laboratory.

DISCUSSION

The non-narcotic analgetics alleviated the antidiuretic response to pain. In all cases this occurred within thirty minutes after administration of the drugs. Fatalities (approximately 50%) occurred with aminopyrine in normally nontoxic doses. In such cases, death occurred within twenty minutes after administration of the drug. Whether hydration or increased absorption rate played a role in this effect is a subject for further investigation.

It appears that the non-narcotic analgetics probably through stimulation of the adrenal-pituitary system or direct cortical stimulation antagonize the antidiuretic response to pain. It seems that the antidiuretic reaction with a concomitant increase in the antidiuretic substance of the blood, is one of the earliest responses occurring from exposure to a noxious stimulus. It may be a part of a generalized metabolic response which is set in motion by pain to the organism. The phenomenon may signify a metabolic rearrangement creating a shift in hormone balance. This is also indicated by the greatly diminished total nonprotein sulfhydryl content of the liver. Substantiating this belief is the general agreement among investigators that glutathione is involved in the intermediary metabolism of carbohydrates and fats and that during stress or pain the reduced glutathione may be converted to oxidized glutathione.

It was not revealed by this study whether hind-leg ischemic pain activates some common mechanism in rats which caused the resultant diminution in TNPSH concentration of the liver and the increase in the ADS titer of the serum. Irrespective of the exact mechanism or mechanisms by which these two responses are produced, both were prevented by non-narcotic analgetics.

SUMMARY

1. Certain non-narcotic analgetics alleviate the pain-induced antidiuresis in the hydrated rat.

2. It has been shown that these effects on diuresis are due to the ADS titer of the serum.

3. Hydration appears to increase the toxicity, especially of aminopyrine.

4. Acetylsalicylic acid 600 mg./Kg. (orally, divided dose) blocks the pain-induced TNPSH lowering of rat liver.

5. Further investigations are being made to determine the specificity of these agents in blocking these responses.

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Composition of Gum Turpentine of Pines XXVII.*

A Report on Three Mexican Pines: *Pinus pringlei* and *P. michoacana* from Michoacan and *P. arizonica* from Chihuahua*

By P. M. ILOFF, Jr., and N. T. MIROV

The gum turpentine of *Pinus pringlei* contained: 73% *d*- and *dl*-α-pinene; 2% *l*-β-pinene; 11% *d*-Δ^3-carene; 1% terpinolene; 2.5% methyl chavicol; 6% *d*-longifolene; and 4.5% pot residue and loss. The gum turpentine of *P. michoacana* contained: 29% *d*- and *dl*-α-pinene; 60% *l*-β-pinene; 1–2% *l*- and *dl*-limonene; 2–3% methyl chavicol; 3% *d*-longifolene; and 4.5% pot residue and loss. The gum turpentine of *P. arizonica* contained: 60% *d*- and *dl*-α-pinene; 29% *l*-β-pinene; 5% *d*-Δ^3-carene; 4.2% tails; and 1.8% pot residue and loss.

PINUS PRINGLEI SHAW

Pinus pringlei, according to Shaw (2), belongs to the group *Insignes*, characterized by the cones that remain closed after the seeds are ripe. It is related to *P. oocarpa*.

Pinus pringlei is a subtropical Mexican species growing in the States of Michoacan, México, Guerrero, and Oaxaca, commonly at altitudes of 1,750 to 1,850 meters above sea level.

The sample of oleoresin used in the present experiment was obtained from Uruapan, Michoacan, through the courtesy of M. Louis Huguet, Forestry Expert of the Food and Agricultural Organization's Mexican project.

EXPERIMENTAL

To distill the turpentine, a batch of 2,989 Gm. of oleoresin of *Pinus pringlei* was heated under reduced pressure so that at the end of distillation, temperature reached 190° and the pressure was re-

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duced to 2 mm. of mercury. Yield of turpentine was 21 per cent of the weight of the oleoresin.

The crude turpentine of *Pinus pringlei* had the following characteristics: $d_4^{20°}$, 0.8642; $n_D^{15°}$, 1.4721; $[\alpha]_D^{20}$, +39.5°. A batch of 596 Gm. of the turpentine was fractionated in a 90-cm. long, 25-mm. inside-diameter Todd column packed with 3/32-inch single turn glass helices. A reflux ratio of 10:1 was maintained. The results of the distillation are shown in Table I.

Judging from their physical properties, fractions 2 and 3 contained mainly *d*- and *dl*-α-pinene. A small yield of *dl*-α-pinene nitrosochloride was prepared from fraction 2. After two recrystallizations from chloroform by addition of cold methanol, the nitrosochloride melted at 103.5–104.5°, and was not depressed in melting point by admixture of authentic *dl*-α-pinene nitrosochloride.

Fractions 4 to 7 were transition fractions containing mostly *d*- and *dl*-α-pinene with some *l*-β-pinene and *d*-Δ^3-carene.

The minimum in optical rotation and maximum in density in fraction 9 indicated the possible presence of *l*-β-pinene. Ten grams of fraction 9 was

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINE OF PINUS PRINGLEI, P. MICHOACANA, AND P. ARIZONICA FROM MEXICO

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
	Pinus pringlei (596 Gm. used) →			d_4^{23}	n_D^{15}	$[\alpha]_D^{23}$
1	760	126–154	0.5	0.856	1.4663	...
2	760	154–156.5	64.5	0.854	1.4668	+47.0
3	760	156.5–158	6.7	0.854	1.4672	+44.4
4	760	158–160	1.0	0.854	1.4689	+36.1
5	760	160–161	0.4	0.855	1.4697	+31.4
6	760	161–163.5	0.5	0.855	1.4705	+27.4
7	760	163.5–164.5	0.5	0.856	1.4718	+21.4
8	14	56–57	1.1	0.859	1.4732	+13.9
9	14	57–58	2.2	0.859	1.4742	+10.5
10	14	58–60	6.2	0.857	1.4745	+12.5
11	14	60–62	0.9	0.854	1.4768	+10.5
12	14	62–65	0.7	0.852	1.4788	+8.2
13	14	65–68	0.5	0.853	1.4812	+6.1
14	14	68–72	0.5	0.854	1.4841	+4.5
15	14	72–78	0.4	0.860	1.4871	+2.6
16	14	78–94	0.7	0.894	1.4962	+3.8
17	14	94–98	1.4	0.950	1.5131	+5.5
18	14	98–108	0.8	0.955	1.5131	+11.1
19	14	108–117	0.3	0.945	1.5068	+32.0
20	14	117–121	0.6	0.937	1.5020	+28.6
21	14	121–122	1.0	0.934	1.5022	+46.2
22	14	122–122.5	2.4	0.932	1.5037	+49.0
23	14	122.5	1.8	0.930	1.5048	+48.0
Pot residue			2.2			
Loss			2.2			
	Pinus michoacana (691 Gm. used) →			d_4^{24}	n_D^{32}	$[\alpha]_D^{24}$
1	760	155	0.1	...	1.3820	...
2	760	155–156	0.2	0.856	1.4622	+8.4
3	760	156–158	30.4	0.857	1.4674	+5.8
4	760	158–160	2.0	0.859	1.4689	+1.9
5	760	160–161	0.6	0.860	1.4712	−3.9
6	760	161–162	1.6	0.860	1.4723	−8.4
7	760	162–163	8.4	0.862	1.4734	−10.8
8	760	163–164	3.9	0.865	1.4752	−15.6
9	760	164–165	15.6	0.865	1.4768	−19.8
10	760	165	13.4	0.866	1.4776	−22.2
11	760	165	9.7	0.866	1.4781	−22.7
12	14	51–52	2.0	0.866	1.4781	−22.7
13	14	52–53	0.6	0.864	1.4783	−22.2
14	14	53–55	0.3	0.859	1.4777	−21.0
15	14	55–61	0.2	0.853	1.4777	−20.4
16	14	61–63	0.4	0.842	1.4773	−19.0
17	14	63–64	0.3	0.841	1.4774	−16.9
18	14	64–66	0.1	0.841	1.4773	−15.5
19	14	66–67	0.3	0.843	1.4784	−13.3
20	14	67–94	0.3	0.873	1.4798	−6.9
21	14	94–98	1.2	0.944	1.5052	−1.5
22	14	98–107	0.6	0.951	1.5076	+1.3
23	14	107–114	0.4	0.945	1.5045	+12.9
24	14	114–116	0.4	0.940	1.5025	+27.2
25	14	116–120	0.8	0.937	1.5020	+40.0
26	14	120–121	1.7	0.929	1.5028	+44.8
Pot residue and loss			4.5			

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
	Pinus arizonica (882 Gm. used) →			d_4^{24}	n_D^{23}	$[\alpha]_D^{24}$
1	760	to 154	0.4	0.858	1.4623	...
2	760	154–155	0.2	0.854	1.4622	...
3	760	155–156	29.6	0.854	1.4652	+20.5
4	760	156–157	17.0	0.854	1.4659	+18.3
5	760	157–159	8.0	0.855	1.4677	+15.5
6	760	159–161	5.2	0.857	1.4693	+8.3
7	760	161–162	4.6	0.859	1.4706	+3.7
8	760	162–164	2.7	0.860	1.4735	−6.9
9	760	164–165	4.1	0.862	1.4751	−12.2
10	760	165–166	12.4	0.863	1.4760	−14.9
11	16	56–57	3.1	0.864	1.4769	−18.1
12	16	57–59	1.9	0.862	1.4752	−7.5
13	16	59–62	1.9	0.858	1.4738	+2.7
14	16	62–63	1.4	0.857	1.4730	+8.8
15	16	63–64	1.2	0.854	1.4738	+2.7
16	16	64–66	0.5	0.852	1.4752	−1.9
17	16	66–68	0.4	0.850	1.4770	−4.2
18	16	68–70	0.2	0.849	1.4785	−6.4
19	16	70–74	0.4	0.850	1.4811	−6.6
20	16	74–77	0.3	0.852	1.4828	−4.2
21	16	77–85	0.3	0.887	1.4830	−2.5
22	16	85–87	0.5	0.867	1.4704	−4.4
23	16	87–91	0.3	0.879	1.4691	−4.1
24	16	Above 91	1.6	0.937	1.4730	−0.8
Pot residue and loss			1.8			

oxidized with alkaline permanganate (3). The sodium salts (0.3 Gm.) received were recrystallized fractionally; the second crop consisted of about 0.1 Gm. of crystals similar in form to authentic sodium nopinate. These crystals were converted to nopinic acid which was recrystallized from water. A few mg. of white crystals (m. p. 125.5–126.5°) were received. Admixture of authentic nopinic acid did not depress the melting point.

Its odor and physical properties indicated that fraction 10 consisted mainly of *d*-Δ^3-carene. From 5 Gm. of fraction 10, about 0.3 Gm. of crude nitrosate was formed. The nitrosate was recrystallized from chloroform by addition of cold methanol. The nitrosate then melted at 145–146° and the melting point was not depressed by admixture of an authentic nitrosate of *d*-Δ^3-carene.

Judging from their physical properties, fractions 11 and 12 were mainly *d*-Δ^3-carene.

Fractions 13 and 14 were transition fractions containing both carene and terpinolene.

Fraction 15 had properties close to those of terpinolene:

	b. p.°	n_D	d_4	$[\alpha]_D$
Fraction 15	72–78 (15 mm.)	1.4871^{23}	0.860^{23}	+2.6
Terpinolene[1]	185 (760 mm.)	1.4864^{25}	0.8560^{25}	0.0

From 2 Gm. of fraction 15, 0.5 Gm. of crude crystalline tetrabromide was prepared. After several recrystallizations from ethyl acetate at 0°, a few mg. with m. p. 115–116° were received. The melting point was not depressed by admixture of authentic terpinolene tetrabromide.

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Fraction 16 was a transition fraction containing both terpenes and oxygenated compounds.

Fractions 17 and 18 had high refractive indexes and densities which indicated the possible presence of methyl chavicol. A 10-Gm. sample of fractions 17 and 18 was oxidized with acid permanganate and 500 mg. of crude homoanisic acid, m. p. 79.5–81°, was received. After two recrystallizations from water, it melted at 85°; the melting point was not depressed by admixture of authentic homoanisic acid.

The physical properties of fractions 19 to 23 indicated that they consisted mainly of a sesquiterpene. From 3 ml. of fraction 22, 2.0 Gm. of a crude crystalline hydrochloride was prepared. After two recrystallizations from glacial acetic acid, 0.8 Gm. of hydrochloride with m. p. 58.6–59.5° was received. The melting point was not depressed by admixture of the hydrochloride of *d*-longifolene.

PINUS MICHOACANA MARTINEZ

In Shaw's monograph (2), *Pinus michoacana* Martinez is included with *Pinus montezumae*, a heterogeneous complex, whose further study, Shaw said, "would be a valuable contribution to science." Since Shaw's time a great deal of work has been done in studying the *Montezumae* complex, but still more should be done.

Pinus michoacana was given the rank of an independent species by Martinez in 1944. This species includes two varieties and two forms. Its typical form occurs in Michoacan, Jalisco, Oaxaca. In Michoacan it may grow with *P. montezumae*, and possibly these two pines cross although no definite information is available on this subject.

A sample of oleoresin of this pine was sent to us by M. Louis Huguet, from Uruapan, Michoacan.

The turpentine was distilled from the oleoresin under reduced pressure so that at the end of distillation, the pressure was 0.1 mm. of mercury and temperature was 190°. Yield of turpentine was 26% of the weight of the oleoresin. The turpentine possessed the following physical characteristics: d_4^{23}, 0.8657; n_D^{21}, 1.4760; $[\alpha]_D^{23}$, −8.4°.

A batch of 691 Gm. of the turpentine was distilled in a 90-cm. long, 25-mm. inside-diameter Todd column packed with 3/32-inch single turn glass helices. Results are shown in Table I.

The properties of fraction 3 are close to those of α-pinene. Both the refractive index and the density indicate the presence of a small amount of β-pinene. Seven milliliters of fraction 3 were used to prepare *dl*-α-pinene nitrosochloride which, after three recrystallizations from chloroform by addition of cold methanol, weighed 1.2 Gm. and melted at 103–104°. The melting point was not depressed by admixture of authentic *dl*-α-pinene nitrosochloride.

The properties of fractions 4 to 7 indicated that they consisted of varying quantities of α- and β-pinene.

The physical properties of fractions 8 to 12 were close to those of *l*-β-pinene. A 20-Gm. portion of fraction 11 was oxidized to sodium nopinate. The sodium nopinate was converted to 1.7 Gm. of nopinic acid which, after one recrystallization from benzene, melted at 125.5–126.5°. The melting point was not depressed by admixture of authentic nopinic acid.

The densities of fractions 12 to 16 approached density of limonene, but the refractive indexes were high.

Fraction 17 appeared to contain mostly *l*- and *dl*-limonene. From 1.9 Gm. of fraction 17, 323 mg. of a tetrabromide, melting at 124–125°, was prepared. The melting point of the tetrabromide was not depressed by admixture of authentic *dl*-limonene tetrabromide.

Fractions 18, 19, and 20 were transition fractions and had properties indicating they were a mixture of terpenes with methyl chavicol.

Fractions 21 and 22 had a high density and refractive index and an odor indicating the presence of methyl chavicol. A 10-Gm. sample of fractions 21 and 22 was oxidized with acid permanganate (1) and 138 mg. of crude white crystals was isolated. After one recrystallization (Darco) from water, the crystals melted at 84–85° and the melting point was not depressed by admixture of an authentic sample of homoanisic acid.

Fractions 23 and 24 had the properties of mixtures of sesquiterpene and methyl chavicol.

Fractions 25 and 26 had properties indicating the presence of *d*-longifolene. From 3 ml. of fraction 26, 1.0 Gm. of sesquiterpene hydrochloride melting at 58–59° was prepared. The melting point of the hydrochloride was not depressed by admixture of a sample of the hydrochloride of *d*-longifolene.

PINUS ARIZONICA ENGELMANN

Pinus arizonica Engelmann was discovered in Arizona, but its main range is in the Mexican states of Sonora, Chihuahua, and Durango. Its variety, *stormiae* Martinez, occurs in the desert mountains of Coahuila and adjacent parts of Nuevo Leon. Shaw (2) considers *P. arizonica* as a variety of *P. ponderosa*. Our findings reported in this paper seem to indicate also that these two pines are closely related.

Oleoresin used in the present investigation was received through the courtesy of Sr. Mario Gonzáles Múzquiz of the Aserraderos Gonzáles Ugarte S. A., a leading lumber company of Chihuahua. Thanks are also due to Ing. Alfredo Parra R. for supervising the collection of this oleoresin. The sample was accompanied with branches, pieces of bark, and cones, which are deposited in our herbarium. The oleoresin was collected at El Retiro, near San Juanito, Chihuahua, at an altitude of 2,500 meters above sea level.

A batch of the oleoresin, weighing 3,628 Gm., was heated under reduced pressure so that at the end, when all turpentine was distilled off, the pressure was 2 mm. of mercury and temperature reached 180°. The remaining rosin was hard and brittle. The turpentine obtained by this means amounted to 908 Gm., or 25% of the weight of the oleoresin.

The turpentine of *P. arizonica* had the following characteristics: d_4^{24}, 0.8585; n_D^{24}, 1.4690; $[\alpha]_D^{24}$, +9.7°.

A batch of 882 Gm. of the turpentine was distilled through a 90-cm. long, 25-mm. inside-diameter, Todd column, packed with 3/32-inch single turn glass helices, and equipped with magnetic control of reflux. A reflux ratio of 10:1 was maintained. Results of the distillation are shown in Table I.

The physical properties of fraction 3 indicated that it was mainly *d*- and *dl*-α-pinene. A nitrosochloride was prepared from 7 ml. of fraction 3. After four recrystallizations from chloroform by addition of cold methanol, 0.7 Gm. of crystals, melting at 104–105°, was received. No depression in melting point was observed on admixture of authentic *dl*-α-pinene nitrosochloride.

Judging from the physical constants, fractions 4 and 5 were also mainly α-pinene and fractions 6 to 9 contained various proportions of α- and β-pinene.

The constants of fractions 10 and 11 indicated that they were mainly *l*-β-pinene. From alkaline permanganate oxidation of 10 Gm. of fraction 11, 0.7 Gm. of nopinic acid, m. p. 126–126.5°, was received. The melting point was not depressed by admixture of authentic nopinic acid.

Fractions 12 and 13 were transition fractions containing both *l*-β-pinene and *d*-Δ^3-carene. The physical properties indicated that fraction 14 was mainly *d*-Δ^3-carene. From 5 Gm. of fraction 14, 1.0 Gm. of crude nitrosate was prepared. On recrystallization from chloroform by addition of cold methanol, 0.6 Gm. of crystals, which melted at 144–145°, was received. The melting point was not depressed by admixture of authentic nitrosate of *d*-Δ^3-carene.

Fractions 16 to 24 contained mixtures of terpenes and possibly some oxygenated compounds which were not identified.

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Prediction of Stability in Pharmaceutical Preparations II.*

Vitamin Stability in Liquid Multivitamin Preparations

By EDWARD R. GARRETT

The physicochemical procedures for prediction of stability at shelf temperatures by rate studies conducted at elevated temperatures have been applied to the vitamin components of liquid multivitamin preparations. The procedures have been expanded to include statistical techniques to provide estimates of error in assays, the extent of confidence to be held in prediction, and recommendations of concentration adjustment to meet minimum assay specifications of the marketed preparations. The validity of the predictions and recommendations has been demonstrated by the agreement of classical stability studies on preparations stored at room temperature with the selected values.

A PROCEDURE for prediction of thermal stability of a component in a complex pharmaceutical preparation was recently outlined (1). This procedure was based on the reproducibility of a degradation rate function at various elevated temperatures and extrapolation of the Arrhenius relation to obtain rate constants that would describe degradation rates at marketing temperatures.

This method has now been applied to many components in complex multivitamin preparations and predictions of stability at room temperature were made in a relatively brief time. Recommendations for changes in initial compositions were made on the basis of specified minimum concentrations to be maintained for a year.

The validity of the predictions and recommendations has been demonstrated by the agreement of classical assays of the shelved preparations with the predicted values.

Statistical procedures are outlined to consider the error in assay and to specify the degree of confidence to be held in prediction. These procedures are most necessary when the assay is subject to wide variation as with biological assays.

Although it is not the purpose of these techniques to evaluate mechanisms of degradation since the primary aim is prognosis and not diagnosis, consideration of the evaluated thermodynamic quantities does permit judicious speculation.

The proper application of this predictive method based on fundamental physicochemical principles should result in the quick release on the market of products with economically and pharmaceutically optimum amounts of vital components.

EXPERIMENTAL

The thermal degradations of components of a multivitamin preparation were studied by assaying the components after various times of maintenance at elevated temperatures. Fifty cubic centimeter aliquots of the preparation were placed in 60-cc. amber, square, screw-cap bottles with vinylite lined caps and incubated at 70, 60, 50, and 4°. The mineral oil constant temperature baths had a probable maximum variation of ±0.25°.

The sample bottle to be assayed was removed from the bath at 8:00 a. m. every morning on the scheduled day of assay and immediately given to the control laboratory. If the assay was not done at the time of sampling, the sample was immediately refrigerated. The preliminary assay schedule was established as, daily for 70°, every other day at 60°, and every third day for 50°. Sampling times were rescheduled on the basis of assay values received to obtain the best distributions of data on assay-time plots. Refrigerated samples (4°) were assayed weekly and no significant changes were noted for the duration of the study.

The product (A) was prepared so that each 5 cc. theoretically contained 1.35 mg. thiamine hydrochloride U. S. P., 0.575 mg. vitamin B_6 hydrochloride, 10.5 mg. nicotinamide U. S. P., 120 mg. ascorbic acid U. S. P., 1.05 mg. riboflavin U. S. P., 0.375 mg. folic acid U. S. P., 3.3 mg. *d*-pantothenyl alcohol, 6.82μg vitamin B_{12} U. S. P., 5,750 units of vitamin A palmitate in oil, 1,080 units of vitamin D_3 in oil and flavoring agents. This preparation contained 36% sugar granulated U. S. P. and 2.35% alcohol. Tragacanth powder U. S. P., was used as a thickening and dispersing agent. The *p*H was 3.2 and no change in *p*H was noted throughout the study. Standard procedures were used in the assay of ascorbic acid (2), thiamine hydrochloride (3), vitamin B_{12} (4), vitamin A (5), folic acid (6), and *d*-pantothenyl alcohol (7).

Another lot (B) was prepared of the same composition as A and also studied for room temperature shelf life stability to test the predictions based on preparation A's accelerated temperature studies. A modified lot (C) was prepared with increased in-

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The cooperation of Mr. Victor F. Dargitz of the Pharmaceutical Development Department of The Upjohn Company for his formulation of the preparations and submission of samples for assay is gratefully acknowledged. Grateful recognition is also given to the Control Division of The Upjohn Company for the vitamin assays.

itial theoretical concentrations of some components, viz., in each 5 cc. were 0.563 mg. folic acid, 4.95 mg. *d*-pantothenyl alcohol and 7,000 U. S. P. units of vitamin A.

CALCULATIONS

The data (y) obtained from assay of the multivitamin preparations which were maintained at the selected temperatures are plotted against time. Examples are given in Fig. 1, 3, and 6. Linearity of such a plot indicates a constant degradation rate independent of concentration, i.e. pseudo zero order rate as

$$dy/dt = -k_0 \qquad (1)$$

The equations of such linear plots would be

$$y = y_0 - k_0 t \qquad (2)$$

where y_0 is the initial concentration of the assayed component, y is the concentration at any time (t), and k_0 is in units of concentration per unit time. Examples of such pseudo zero order thermal degradation are ascorbic acid above concentrations of 70 mg./5 cc. in Fig. 1, the initial values of the plots for folic acid in Fig. 3 and for vitamin A in Fig. 6; especially at the lower temperatures in the latter two cases.

Concentration.—Time plots are not always linear, either for part or all of the degradation study. The data must frequently be treated as a pseudo first order rate dependent on the concentration of the component under assay as

$$dy/dt = -k_1' y \qquad (3)$$

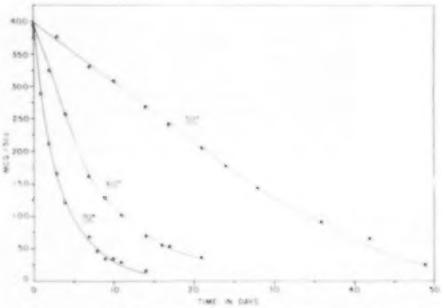

Fig. 3.—The thermal degradation of folic acid in a liquid multivitamin preparation (A); μg./5 cc. against time in days.

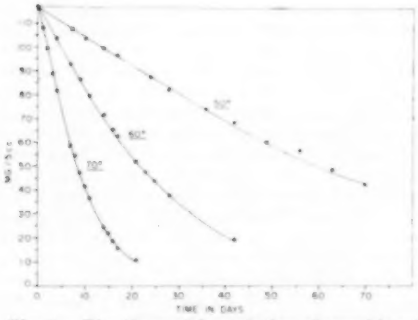

Fig. 1.—The thermal degradation of ascorbic acid in a liquid multivitamin preparation (A); mg./5 cc. against time in days.

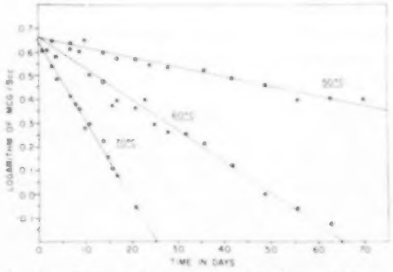

Fig. 4.—First order plots of the thermal degradation of vitamin B_{12} in a liquid multivitamin preparation (A); logarithms of concentration (μg./5 cc.) against time in days.

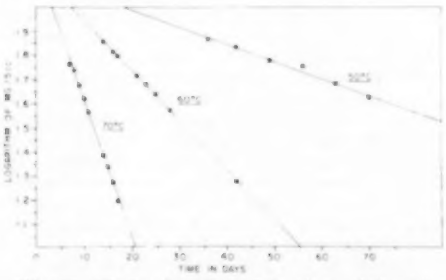

Fig. 2.—First order plots of the thermal degradation of ascorbic acid in a liquid multivitamin preparation (A); logarithms of concentration (mg./5 cc.) against time in days.

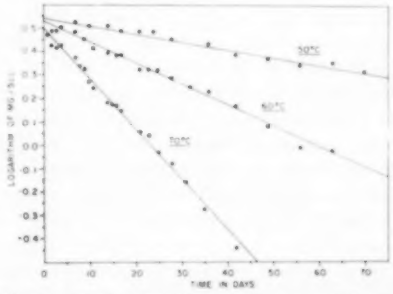

Fig. 5.—First order plots of the thermal degradation of *d*-pantothenyl alcohol in a liquid multivitamin preparation (A); logarithms of concentration (mg./5 cc.) against time in days.

The integrated form of this expression would be

$$\log y = \log y_0 - (k_1'/2.303)t \qquad (4)$$

or

$$\log y = \log y_0 - k_1't \qquad (5)$$

where k_1' is in reciprocal time units and k_1 is in reciprocal time units divided by 2.303.

When plots of the logarithm of the concentration ($\log y$) against time (t) are linear, the rates are pseudo first order with slopes of k_1. Examples are given in Fig. 2, 4, and 5.

In many cases, the order of the degradation rate (pseudo zero or pseudo first) can be chosen on the basis of inspection of the data for part or all of the time study. In all cases of thermal degradation studied so far, these two rate orders have served satisfactorily to characterize the loss of assay potency within the errors of assay.[2] However, expressions can be readily derived by analogous methods for partial or higher order reactions and degradations.

Since many of the component assays are based on biological methods with relatively large inherent error, it is important that statistical techniques be employed to determine the best estimates of rate constants and errors in rate constants and in assay. This is done by the method of least squares (9). Rate constants (k_0 and k_1) and their standard deviations (σ_{k_0} and σ_{k_1}) determined by this method on n experimental points are tabulated in Table I for the pseudo zero order degradations and in Table II for the pseudo first order degradations.

This method of least squares also allows estimates of the error in the assay (σ_y) or in the logarithm of assay values ($\sigma_{\log y}$). The 95% confidence limits of a single assay or log assay may be estimated by doubling these values.[3] These data are tabulated in Table III. The agreement in estimates of error from statistics of the zero and first order plots and dilution studies[4] is excellent.

Linear correlations between rates and temperatures can be made by use of the logarithmic form of the Arrhenius equation:

$$\log k = P - (\Delta H_a/2.303\ R)\,(1/T) \qquad (6)$$

where k is the rate constant, R is the gas constant (1.987 calories degree^{-1} mole^{-1}), P is a constant associated with entropy of the reaction and/or collision factors, ΔH_a is defined as the heat of activation and T is the absolute temperature (273 + °C.). A simplified form of equation (6) is

$$\log k = P - S/T \qquad (7)$$

If such a relation among several values determined at elevated temperatures and the absolute temperature is reasonably linear, the prediction of stability at shelf temperatures should be practical.

The heats of activation (ΔH_a) and other data derived from application of the Arrhenius expressions of equations (6) and (7) to the plots of $\log k$ against the reciprocal of the absolute temperature ($1/T$) are tabulated in Table IV. A typical plot is given in Fig. 7, where the open circles represent the range of standard deviation of an experimentally determined rate constant and the horizontal lines above and below the datum represent its 95% confidence limits.

The data are fitted by the statistical methods of least squares[5] which permit the estimates of the standard error (σ_s) of the slope (S) of equation (7) and of the standard deviation ($\sigma_{\log k}$) of the logarithm of a rate constant, which statistics are also tabulated in Table IV.

The values of P and S of Table IV may be substituted into equation (7) to predict the logarithm of a rate constant ($\log k$) at a lower temperature. Of course, the $\log k$ value may also be read from the extrapolated Arrhenius plot. The estimated error in prediction (σ_p) of a $\log k$ at an absolute temperature (T_p) may be based (13) on the equation:

$$\sigma_p = \sigma_{\log k}\sqrt{(N+1)/N + [1/T_p - (1/T)_a]^2/\Sigma[1/T - (1/T)_a]^2}$$

where $\sigma_{\log k}$ and N are tabulated in Table IV, $(1/T)_a$ is the mean of the reciprocals of the absolute temperatures at which the rates were eval-

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[2] There may be instances of difficulty in assigning order of degradation rate and more elegant statistical techniques may be necessary (8). A simple statistical test would be to fit the data by least squares (9) to both zero order and first order plotting and determine the residual variance (σ^2, square of standard deviation) about both regression lines. This measures the variation in excess of the expected linearity in the plots of concentration or logarithm of concentration against time. The variance (σ_y^2) about the regression, concentration on time, may be compared to the variance ($\sigma_{y'}^2$) derived from the variance ($\sigma^2_{\log y}$) about the regression, logarithm concentration on time, by:

$$\sigma_{y'}^2 = (2.303\ y)^2\sigma^2_{\log y}$$

Whichever variance (σ_y^2 or $\sigma_{y'}^2$) is in excess is most probably derived from the least valid linear relation. The factor, y, in the above equation may be estimated by the average concentration value during the rate study. Statistically significant difference may be tested by the "F" test (10).

[3] More accurate estimates may be made by $\pm t\sigma$ where 5% t may be obtained from statistical tables for the appropriate degrees of freedom ($n - 2$) where the appropriate n may be obtained from Tables I and II.

[4] See footnote (c), Table III.

[5] There are several more elegant methods of weighting the ordinates in linear plots so that those values with least error are given greater significance in curve fitting. Procedures have been devised (11) for weighting ordinates (e. g., $\log k$) by their actual error (viz., variance, $\sigma^2_{\log k} = \sigma_k^2/(2.303\ k)^2$ where σ_k or k are tabulated in Tables I and II).

These procedures have been extended on the premise that equal weight or equal estimate of error is given to each assay value obtained throughout a zero order rate study (12). It follows that the variance or error in a rate constant should increase with decreasing temperature for rate studies conducted for the same length of time. On this premise the $\log k$ values at higher temperatures should be given greater weight in fitting the Arrhenius equation. However, our major interest is not in obtaining the best estimate of heats of activation or slope, afforded by these procedures, but is in prediction by extrapolation to lower temperatures.

Such prediction could be based on the valid expectation of linear relation between the logarithm of rate constant ($\log k$) and the reciprocal of absolute temperature ($1/T$). It is not necessary that this relation be rigorous for practical prediction. The data fitted to the Arrhenius expression need only to approximate linearity, as will a small segment of any curve. Statistical "least squares" fitting will give satisfactory estimates of the error in prediction by extrapolation regardless of whether or not the Arrhenius expression is *absolutely* applicable although it has been found valid well within the assay errors in predictive studies.

This practical attitude, however, increases the relative significance of the low temperature rate values that are experimentally obtained. The values adjacent to those to be estimated by extrapolation should have greater weighting than what would be given on the basis of their experimentally estimated error relative to the higher temperature rates. The degree of increase in significance as a function of their proximity to the temperature of the values to be predicted cannot be readily quantitated without exact knowledge of these hypothetically possible deviations from the linear logarithmic Arrhenius equation. Thus it may be just as well to ignore the elegant procedures outlined above and fit the available data on the basis of equal weighting of the ordinates rather than on an equal weighting of the basic assay data of which an ordinate is a function.

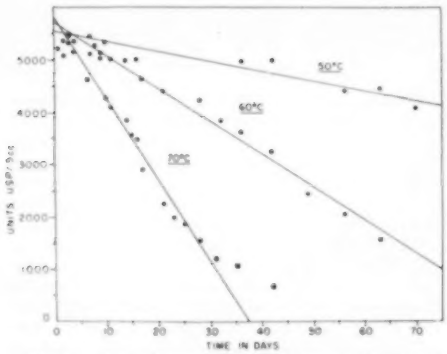

Fig. 6.—The thermal degradation of vitamin A in a liquid multivitamin preparation (A); units U. S. P./5 cc. against time in days.

uated and $\Sigma[1/T - (1/T)_a]^2$ are the sums of squares of the deviations of these reciprocal temperatures from their mean. The degrees of freedom (D. F.) for σ_p are $N - 2$ and 95% confidence limits may be estimated from "t" σ_p where 5% "t" is obtained from statistical tables.[6]

The relatively large decrease in t for the increase in number of temperature studies (N) from 3 to 4 may justify the cost of an additional rate study at another temperature. In general, increased confidence in predicted values from increased numbers of temperature studies must be balanced by the necessary economic considerations.

Predicted rates and times of maintenance of desired minimum values for assumed initial assay values of the components of the preparations are given in Table V for 30 and 25°. In addition the estimated standard error of the predicted rates and times of maintenance as derived from fitting the Arrhenius relation are given. A typical plot is shown in Fig. 7 where the standard error in a predicted rate is shown by the height of the rectangles at 30, 25, and 20°.

The predicted rate constants (k_0 or k_1) are substituted into the appropriate zero or first order equations (2) and (5), respectively. For a given initial assay value (y_0) and a specified minimum assay value ($y = y_m$) to be maintained, the time of maintenance ($t = t_m$) of this minimum assay value can be computed as in Table V. The same equations may be used to determine the necessary initial assay (y_0) to maintain the minimum assay value specified ($y = y_m$) for a desired time interval ($t = t_m$), where one year at 30° may be a safe margin of maintenance.

DISCUSSION

If a one year maintenance at room temperature of the stated minimum assay values in Table V is desired, the initial amounts of folic acid, *d*-pantothenyl alcohol and vitamin A in the preparation A, may be insufficient.[7]

This is especially true if it is specified that the minimum assay value must be maintained at 30° using the maximum standard error in a predicted rate and considering the 95% confidence limits of an individual assay. This conservative attitude may be the practical one if the product is to be spot tested for stated minimum value of a component after a year's market shelf life. Initial assay values to meet confidently these criteria for maintenance of minima are calculated as specified in this paper and would be 560 μg/5 cc. of folic acid, 4.95 mg./5 cc. of *d*-pantothenyl alcohol, and 7,000 U. S. P. units/5 cc. of vitamin A.

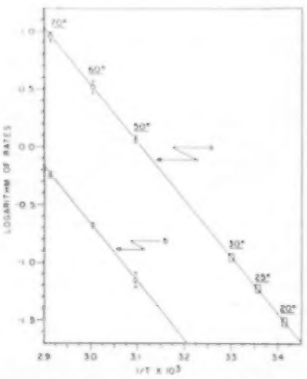

Fig. 7.—Arrhenius plots for ascorbic acid. Curve *a* is the plot of the logarithm of the slopes (mg./5 cc./day) of zero order plots against the reciprocal of the absolute temperature (T) for the thermal degradation of ascorbic acid above 70 mg./5 cc. in liquid multivitamin preparation A. Curve *b* is a similar plot of the logarithm of ten times the slopes of first order plots (in days) for the thermal degradation of ascorbic acid below 70 mg./5 cc. in the same preparation A. The circles represent the standard deviation of an experimental log rate and the rectangles the standard deviation of a predicted log rate. The horizontal lines represent the 95% confidence limits of the experimental log rates.

The validity of these predictions of stability in such pharmaceutical preparations is clearly shown in the typical Fig. 8–11. These are plots of the classically performed assays of preparations to evaluate room temperature shelf life. The solid lines represent the predicted thermal degradation of the component at the given temperatures. The dashed lines encompass the standard error in the predicted thermal degradations and 95% confidence limits of a single assay. It is expected that the assay values of preparations maintained at room temperature will fall within these 25 and 30° curves and the estimated error, which they do. The data from the flushed preparations, i. e., with increased initial amounts of components to compensate for

[6] The 5% "t" is 4.3 for $N = 4$, 2 D. F.; is 11.7 for $N = 3$, 1 D.F.; and is indeterminate for $N = 2$, no D.F. For $N = 3$, the temperatures were 50, 60, and 70° and 40° was added for $N = 4$.

[7] If the initial assay values of the other components that appear doubtful in Table V coincide with their theoretical as given in the experimental, they would be satisfactory.

TABLE I.—TABULATION OF SLOPES (k_0)[a] AND THEIR STANDARD DEVIATION (σ_{k0})[b] OF ZERO ORDER PLOTS[c] FOR THERMAL DEGRADATION OF COMPONENTS IN LIQUID MULTIVITAMIN PREPARATIONS AT VARIOUS TEMPERATURES

Component	Units of Concentration	70° k_0	70° σk_0	70° nd	60° k_0	60° σk_0	60° nd	50° k_0	50° σk_0	50° nd
Ascorbic acid[e]	mg./5 cc.	8.96	0.29	5	3.29	0.14	6	1.197	0.025	8
Folic acid[f]	μg./5 cc.	105	*..[g]	2	33.0	0.5	4	9.15	0.15	9
Vitamin A[f]	U. S. P. u./5 cc.	155.4	10.2	17	63.41	2.45	16	19.41	2.27	8

[a] The slope or rate constant, k_0, is in units of concentration/day.
[b] The 95% confidence limits of k may be estimated by $\pm$ "t" σk_0 where 5% "t" may be obtained from statistical tables for the appropriate degrees of freedom ($n - 2$).
[c] Zero order plots are linear plots of concentration (y) against time (t) in days and conform to the equation: $y = y_0 - kt$, where y_0 is the concentration at zero time.
[d] The degrees of freedom, $n - 2$, may be derived from n, the number of experimental points used in fitting the curve.
[e] The thermal degradation of ascorbic acid is pseudo zero order only above 70 mg./5 cc.
[f] The thermal degradation is pseudo zero order only at the beginning of the studies. The extent of the initial zero order degradation increased with decreasing temperature for a constant initial concentration.
[g] A slope (k_0) based on two experimental points (n) leaves no degrees of freedom for estimating error in the slope. If a third datum, subsequent to the initial two used in estimating the k_0 for folic acid had been included, the estimated assay error (residual about regression) would have been significantly different from the assay error estimated from the studies made at the other temperatures. Thus it was assumed that the third datum point deviated from the initial zero order rate.

TABLE II.—TABULATION OF SLOPES (k_1)[a] AND THEIR STANDARD DEVIATION (σ_{k_1})[b] OF FIRST ORDER PLOTS[c] FOR THERMAL DEGRADATION OF COMPONENTS IN LIQUID MULTIVITAMIN PREPARATIONS AT VARIOUS TEMPERATURES

Component	70° 10^2k_1	70° $10^2\sigma k_1$	70° nd	60° 10^2k_1	60° $10^2\sigma k_1$	60° nd	50° 10^2k_1	50° $10^2\sigma k_1$	50° nd
Ascorbic acid[e]	5.78	0.12	9	2.07	0.02	8	7.07	0.38	6
Folic acid[f]	11.01	0.36	7	5.33	0.15	5	24.0	0.5	4
Vitamin B_{12}	3.32	0.10	14	1.308	0.054	18	4.08	0.22	14
Thiamine HCl	2.74	0.08	13	0.866	0.028	18	2.58	0.27	14
d-Pantothenyl alcohol	2.16	0.04	20	0.890	0.023	18	3.20	0.23	14
Vitamin A[f]	2.62	0.02	13	1.38	0.02	5			

[a] The slope or rate constant, k_1, is in units of (2.303 days)$^{-1}$.
[b] The 95% confidence limits of k_1 may be estimated by $\pm$"t" σk_1 where 5% "t" may be obtained from statistical tables for the appropriate degrees of freedom ($n - 2$).
[c] First order plots are linear plots of logarithms of concentration (y) or logarithms of fractions of initial concentration (y/y_0) against time (t) in days and conform to the equation: $\log (y/y_0) = -k_1t$, where y_0 is the concentration at zero time.
[d] The degrees of freedom, $n - 2$, may be derived from n, the number of experimental points used in fitting the curve.
[e] The thermal degradation of ascorbic acid is pseudo first order below 70 mg./5 cc.
[f] The thermal degradation is pseudo first order subsequent to an initial zero order rate. The extent of first order degradation decreased with decreasing temperature for a constant initial concentration.

TABLE III.—TABULATION OF STANDARD DEVIATIONS OF ASSAY VALUES (σ_y)[a] DERIVED FROM ZERO ORDER DEGRADATIONS AND OF STANDARD DEVIATIONS OF LOGARITHMS OF ASSAY VALUES ($\sigma_{\log y}$)[b] DERIVED FROM FIRST ORDER DEGRADATIONS OF COMPONENTS IN LIQUID MULTIVITAMIN PREPARATIONS

Component	Derived from Zero Order Degradations: Approximate Concentration Range of y	Derived from Zero Order Degradations: S. D. of an Assay (σ_y)	Derived from First Order Degradations: Approximate Concentration Range of y	Derived from First Order Degradations: S. D. of log assay ($\sigma_{\log y}$)	Derived from First Order Degradations: Concn. (y)[d] at Which $\sigma_{\log y}$ Corresponds to σ_y from Zero Order
Ascorbic acid	70–120 mg./5 cc.	1.0 mg./5 cc.	10–70 mg./5 cc.	0.010	43 mg./5 cc.
Folic acid[c]	200–400 μg./5 cc.	3.9 μg./5 cc.	0–200 μg./5 cc.	0.021	81 μg./5 cc.
Vitamin B_{12}[c]		0.1 μg./5 cc.	1–5 μg./5 cc.	0.029	1.5 μg./5 cc.
Vitamin A	4,000–6,000 U. S. P. u./5 cc.	260 U. S. P./5 cc.	1,000–4,000 U. S. P./5 cc.	0.0273	4,200 U. S. P./5 cc.
d-Pantothenyl alcohol[c]		0.06 mg./5 cc.	1.0–4.0 mg./5 cc.	0.0188	1.4 mg./5 cc.
Thiamine HCl		0.044 mg./5 cc.	<1.4 mg./5 cc.	0.0193	1.0 mg./5 cc.

[a] Square root of residual variance about regression of assay values (y) on time (t) in days. The variances were pooled from the studies at the various temperatures, i. e., $\sigma_y^2 = \Sigma_i(N_i\sigma^2_{y_i})/\Sigma N_i$ where N_i is the degrees of freedom for the variance of $\sigma_{y_i}^2$ at the i^{th} temperature degradation study.
[b] Square root of residual variance about regression of logarithm of assay values (log y) on time (t) in days. The variances were pooled from the studies at the various temperatures, i. e., $\sigma_{\log y}^2 = \Sigma_i(N_i\sigma^2_{\log y_i})/\Sigma N_i$ where N_i is the degrees of freedom for the variance of $\sigma^2_{\log}$ at the i^{th} temperature degradation study.
[c] A study of the reliability of assay by ten dilutions of the original preparation A with the vehicle at 10% intervals and determining the variance about regression of assay against dilution resulted in estimates of standard deviation for folic acid; $\sigma_y = 3.87$ μg./5 cc.; for vitamin B_{12}, $\sigma_y = 0.097$ μg./5 cc.; and for *d*-pantothenyl alcohol, $\sigma_y = 0.07$ mg./5 cc.
[d] The relationship between the standard deviation (σ_y) of an assay value (y) and the standard deviation ($\sigma_{\log y}$) of the logarithm of the assay value (log y) is: $\sigma_y = 2.303\, y\sigma_{\log y}$. Calculation of y at which the two standard deviations correspond can serve as a valid measure of their equivalence.

TABLE IV.—TABULATION OF HEATS OF ACTIVATION AND OTHER DATA DERIVED FROM ARRHENIUS PLOTS OF LOGARITHMS OF PSEUDO ZERO (k_0) AND FIRST (k_1) ORDER RATE CONSTANTS AGAINST THE RECIPROCALS OF THE ABSOLUTE TEMPERATURES (T)[a]

Component	Units of Rate Constant (k)	P	S	ΔH_a (in Kcal./Mole.)	N[b]	σ_S[c]	$10^3\sigma_{\log k}$[d]
(From Pseudo Zero Order Rates)							
Ascorbic acid[e]	mg./5 cc./day	15.38	4,967	22.8	4	76	15.7
Folic acid[f]	μg./5 cc./day	19.15	5,874	26.9	3	73	9.35
Vitamin A[f]	U. S. P. u./5 cc./day	16.82	5,010	22.9	3	312	39.8
(From Pseudo First Order Rates)							
Ascorbic acid[e]	day^{-1}/2.303	13.50	5,054	23.1	3	19	2.46
Folic acid[f]	day^{-1}/2.303	9.73	3,665	16.8	3	32	4.12
Vitamin B_{12}	day^{-1}/2.303	13.25	5,049	23.1	3	236	0.303
Vitamin A[f]	day^{-1}/2.303	7.72	3,191	14.6	2	[g]	[g]
d-Pantothenyl alcohol	day^{-1}/2.303	11.72	4,589	21.0	3	84	10.8
Thiamine HCl	day^{-1}/2.303	15.00	5,682	26.0	3	14	1.80

[a] The equation of such plots is $\log k = P - S/T = P - (\Delta H_a/2.303\ R)\ (1/T)$ where P is the intercept, S is the slope, ΔH_a is the heat of activation, and R is the gas constant in cal./mole.

[b] The number of temperature studies, N, of a component's thermal degradation may be used to estimate the degrees of freedom ($N - 2$) for estimated error (σ_S) in slope S and the estimated error ($\sigma_{\log k}$) in logarithm of rate constant ($\log k$). The 5% "t" is 4.3 for $N = 4$, 12.7 for $N = 3$ and indeterminate for $N = 2$. The temperatures studied were 50°, 60°, and 70° for $N = 3$ and 40° was added for $N = 4$.

[c] Standard error (σ_S) of slope (S). The 95% confidence limits of S may be estimated by $\pm$ "t" σ_S where 5% "t" may be obtained from statistical tables for the appropriate degrees of freedom ($N - 2$).

[d] Standard deviation ($\sigma_{\log k}$) of logarithms of the rate constant ($\log k$) derived from the square root of residual variance about regression of $\log k$ values on $1/T$. The 95% confidence limits of a single $\log k$ value may be estimated by $\pm$ "t" $\sigma_{\log k}$ where 5% "t" may be obtained from statistical tables for the appropriate degrees of freedom ($N - 2$).

[e] The thermal degradation of ascorbic acid is pseudo zero order above, and pseudo first order below 70 mg./5 cc. in the same preparation.

[f] The thermal degradation is initially pseudo zero order and subsequently first order. The extent of the former increased with decreasing temperature.

[g] A line based on two experimental points allows no degrees of freedom for estimating such standard errors.

TABLE V.—TABULATION OF PREDICTED RATES AND TIMES OF MAINTENANCE OF A STATED MINIMUM ASSAY OF COMPONENTS OF LIQUID MULTIVITAMIN PREPARATION A AT 25° AND 30° C.

Component	Temperature (° C.)	Predicted Rate Constant (k)	Range of Standard Error of Predicted Rate	Years (t_m) of Maintenance of Minimum Assay and Standard Error[b]	Minimum Assay to be Maintained (y_m)	Assumed Initial (y_0)[a]
(From Pseudo Zero Order Rates)[c]						
Ascorbic acid	30°	11.1×10^{-2} mg./5 cc.	$(10.4–11.8) \times 10^{-2}$	1.14 ± 0.07	70 mg./5 cc.	116 mg./5 cc.
	25°	5.9×10^{-2} mg./5 cc.	$(5.5–6.2) \times 10^{-2}$	2.14 ± 0.14		
Folic acid	30°	0.583 μg./5 cc.	(0.551–0.616)	0.59 ± 0.03	250 μg./5 cc.	375 μg./5 cc.
	25°	0.275 μg./5 cc.	(0.259–0.294)	1.25 ± 0.08		
Vitamin A	30°	1.91 U. S. P. u./5 cc.	(1.54–2.42)	0.9 ± 0.2	5,000 U. S. P. u./5 cc.	5,750 U. S. P u./5 cc.
	25°	1.00 U. S. P. u./5 cc.	(0.76–1.32)	1.6 ± 0.4		
(From Pseudo First Order Rates)[d]						
Vitamin B_{12}	30°	3.9×10^{-4}	$(3.3–5.0) \times 10^{-4}$	0.86 ± 0.18	3.41 μg./5 cc.	4.53 μg./5 cc.
	25°	2.1×10^{-4}	$(1.7–2.6) \times 10^{-4}$	1.6 ± 0.4		
d-Pantothenyl alcohol	30°	3.8×10^{-4}	$(3.7–4.0) \times 10^{-4}$	0.30 ± 0.01	3.0 mg./5 cc.	3.3 mg./5 cc.
	25°	2.15×10^{-4}	$(2.1–2.2) \times 10^{-4}$	0.53 ± 0.02		
Thiamine HCl[e]	30°	1.8×10^{-4}	.....	1.08	1.00 mg./5 cc.	1.18 mg./5 cc.
	25°	8.7×10^{-5}	.....	2.24		

[a] In general, these assumed initial assay values are based on the average of the intercepts at zero time of the rate studies at the several temperatures.

[b] The years (t_m) of maintenance of the minimum assay desired are based on the expressions $t_m = (y_0 - y_m)/365\ k_0$ for the pseudo zero order rates and $t_m = (\log y_0 - \log y_m)/365\ k_1$ for the pseudo first order rates.

[c] The pseudo zero order rate constants, k_0, are in the concentration units designated per day.

[d] The units of the pseudo first order rate constants are (2.303 day)$^{-1}$.

[e] The data for the Arrhenius' plot are so colinear that the error about this linearity is negligible.

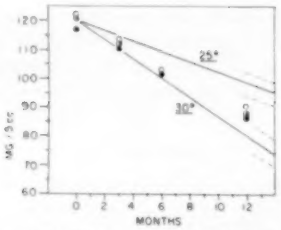

Fig. 8.—Verification of prediction of ascorbic acid stability at room temperature. The solid lines are predicted thermal degradation rates of ascorbic acid in liquid multivitamin preparation A at 25° and 30°. The dashed lines encompass standard error in predicted value and 95% confidence limits of a single assay. The open circles are data from preparation A, the closed circles are data from a similar preparation B and the half closed circles are data from a modified preparation C with increased concentration of some vitamin components.

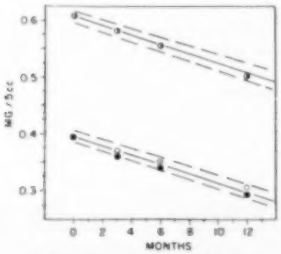

Fig. 9.—Verification of prediction of folic acid stability at room temperature. The solid lines are predicted thermal degradation rates of folic acid in liquid multivitamin preparation A at 25°. The dashed lines encompass standard error in predicted values and 95% confidence of a single assay. The open circles are data from preparation A, the closed circles are data from a similar preparation B, and the half closed circles are data from a modified preparation C with increased initial concentration of folic acid and other vitamin components.

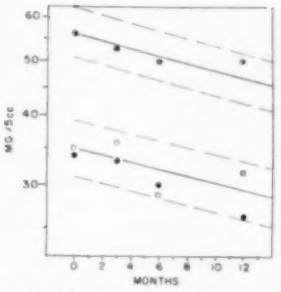

Fig. 10.—Verification of prediction of *d*-pantothenyl alcohol stability at room temperature. The solid lines are predicted thermal degradation rates of *d*-pantothenyl alcohol in liquid multivitamin preparation A at 25°. The dashed lines encompass standard error in predicted values and 95% confidence of a single assay. The open circles are data from preparation A, the closed circles are data from a similar preparation B, and the half closed circles are data from a modified preparation C with increased initial concentration of *d*-pantothenyl alcohol and other vitamin components.

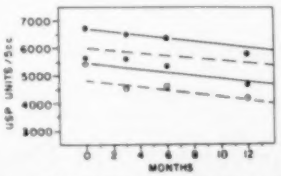

Fig. 11.—Verification of prediction of vitamin A stability at room temperature. The solid lines are predicted thermal degradation rates of vitamin A in liquid multivitamin preparation A at 30°. The dashed lines encompass standard error in predicted values and 95% confidence of a single assay. The open circles are data from preparation A, the closed circles are data from a similar preparation B, and the half closed circles are data from a modified preparation C with increased initial concentration of vitamin A and other vitamin components.

the estimated degradation below specified minima, are also plotted in Fig. 9–11.

It has been noted that in several studies, *viz.*, ascorbic acid, folic acid, and vitamin A, the component has an initial pseudo zero order degradation which subsequently becomes pseudo first order. Frequently the extent of such zero order degradation increases with decreasing temperature. One possible explanation is that at concentrations above the solubility of the material, the degradation rate is constant and pseudo zero order since it is dependent on the concentration of dissolved material which is also constant. Below saturation, the amount of dissolved material decreases with degradation and the rate is pseudo first order or apparently concentration dependent. Solubility may decrease with temperature and thus the extent of pseudo zero order degradation may also increase. Possible justification of this hypothesis may be found in similar heats of activation for both zero and first order segments of the thermal degradation. Thus, ascorbic acid appears to degrade by the same mechanism for both orders since the heats of activation calculated from both are similar as is shown in Table IV and by the parallelism of Arrhenius plots in Fig. 7. This explanation is not satisfactory for folic acid or vitamin A. If one component is complexed by another and thus possibly degraded by several mechanisms, changes in degradation rate order may also occur with changes in concentration. It is possible that these different mechanisms may exhibit different heats of activation.[8]

[8] It should *not* be implied that some components of multivitamin preparations may have similar heats of activation in all such preparations. For example, in one other preparation studied ascorbic acid had a heat of activation of 16.2 Kcal./mol. and thiamine hydrochloride had 13.1 Kcal./mol. These values do not concur with those listed in Table IV for these components in the preparations studied in this paper. It follows that a spectrum of elevated temperatures is necessary rather than one plus an assumed heat of activation for the confident prediction of shelf stability of components in new vehicles.

SUMMARY

(1) The rates of thermal degradation of ascorbic acid, vitamin B_{12}, folic acid, vitamin A, *d*-pantothenyl alcohol, and thiamine hydrochloride in a liquid multivitamin preparation have been studied at elevated temperatures and the orders of degradation determined.

(2) Zero and first order rate constants and estimates of their error and that of assay of the vitamin components have been statistically evaluated.

(3) Heats of activation and their estimated error have been determined by statistical fitting of the rate constants to the Arrhenius equation.

(4) Stability of vitamin components at room temperature has been predicted by work performed in a relatively brief time and the predictions confirmed within the estimated error by room temperature, shelf stability studies. Preparations, with recommended increases in initial concentrations to meet specified minimums after a one year shelf life, degraded at predicted rates.

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Preparation and Properties of 8-Chlorotheophylline Salts of Some Nitrogen Bases*

By D. J. LAMB† and F. W. BOPE

The 8-chlorotheophylline salts of three narcotic nitrogen bases were prepared. Some of their physical and chemical properties were determined including infrared spectra and titration curves. Optical crystallographic properties were investigated in two of the new derivatives.

THE WORK OF CUSIC (1–4) and Robinette and Bope (5) prompted the investigation of the 8-chlorotheophylline salts of several synthetic narcotics including meperidine, levorphan, and metopon. The purpose of this research was the preparation of the above mentioned 8-chlorotheophyllinates and the investigation of some of their more important physical and chemical properties. Synthesis of similar salts of aniline and aminopyrine was not accomplished using the methods tested.

EXPERIMENTAL

The 8-Chlorotheophylline Salt of Meperidine.—Meperidine base was obtained from a 0.300 Gm. (0.0011 mole) sample of the hydrochloride salt by extraction with diethyl ether after making the aqueous solution alkaline with NaOH T. S. The ether extract was then evaporated to a viscous liquid on a steam bath. The resulting liquid was dissolved in 25 ml. of ethanol and this solution was added to a hot suspension of 0.200 Gm. (0.0093 mole) of 8-chlorotheophylline in 25 ml. of ethanol. The temperature of the mixture was maintained at 60–70° for one-half hour during which all the 8-chlorotheophylline went into solution. The volume of the solution was then reduced to 5 ml. by vacuum distillation, maintaining the still temperature at 30–35°. Upon cooling crystallization took place. The crystals were filtered and purified by recrystallization from 99% 2-propanol. The crystals were dried in an Abderhalden drying pistol over phosphorus pentoxide at 100° and 3 mm. pressure for three hours. The melting range was 155–157°. The material did not melt completely but appeared to form two phases consisting of melt and solid. The total yield based on the weight of 8-chlorotheophylline was 28.6%.

* Received June 17, 1955, from the College of Pharmacy, The Ohio State University, Columbus.
Based in part upon a thesis submitted by D. J. Lamb to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree Master of Science.
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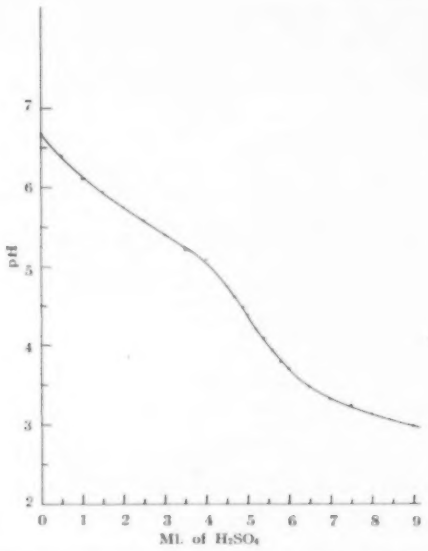

Fig. 1.—Titration curve of meperidine 8-chlorotheophyllinate.

Anal.—Calcd. for $C_{15}H_{21}NO_2 \cdot C_7H_7ClN_4O_2$: C, 57.19; H, 6.10; N, 15.16. Found: C, 56.77; H, 5.98; N, 14.58.

Optical Crystallographic Properties.—The crystals under plane polarized light appeared as elongated rods, showing no pleochroism. Under crossed Nicols, the crystals were found to be anisotropic and showed parallel extinction; no useful interference figures were observed.

Refractive Indexes.—1.538 (lengthwise), 1.615 (crosswise). Both ±0.002.

The 8-Chlorotheophylline Salt of Levorphan.—Levorphan free base was obtained from the tartrate salt by extraction of the aqueous solution with chloroform after making the solution alkaline with sodium carbonate. The chloroform extract was concentrated on a steam bath and the free base crystallized on cooling.

A 0.238 Gm. (0.0009 mole) sample of levorphan base and 0.171 Gm. (0.0008 mole) of 8-chlorotheophylline were added to 5 ml. of 99% 2-propanol and the mixture was heated to 70°. Six drops of water were added with mixing to effect complete solution. The temperature of the solution was maintained at 70–75° for one-half hour. Upon cooling crystallization took place. The crystals were filtered, recrystallized from 2-propanol, and dried in the manner described above. The melting range was 245–248° with decomposition. The yield based on the weight of 8-chlorotheophylline was 64%.

Anal.—Calcd. for $C_{17}H_{23}NO \cdot C_7H_7ClN_4O_2$: C, 61.06; H, 6.40; N, 14.83. Found: C, 61.00; H, 6.31; N, 15.25.

Optical Crystallographic Properties.—The crystals under plane polarized light appeared as rods and showed no pleochromism. Under crossed Nicol prisms, the crystals were found to be anisotropic and showed parallel extinction; no useful interference figures were observed.

Refractive Indexes.—1.648 (lengthwise), 1.561 (crosswise). Both ±0.002.

The 8-Chlorotheophylline Salt of Metopon.—Metopon free base was obtained by extraction of an aqueous solution of the hydrochloride salt with chloroform after making the solution alkaline with sodium carbonate T. S. The chloroform extract was concentrated on a steam bath and the free base crystallized from solution.

A 0.208 Gm. (0.00069 mole) sample of metopon base and 0.148 Gm. (0.00068 mole) of 8-chlorotheophylline were added to 10 ml. of 99% 2-propanol and the mixture was heated to 70°. Six drops of water were added to effect complete solution and the temperature of the solution was maintained at 70–75° for one-half hour. Upon cooling a granular material precipitated. Attempts were made to crystallize the material using 2-propanol and absolute ethanol as solvents but only granular material was obtained. This material was dried in the manner described above. Melting range was 171–175°. The yield based on the weight of 8-chlorotheophylline was 47%.

Anal.—Calcd. for $C_{18}H_{21}NO_3 \cdot C_7H_7ClN_4O_2$: C, 58.41; H, 5.49; N, 13.62. Found: C, 57.94; H, 5.94; N, 12.20.

Molecular Proportion.—The new derivatives of 8-chlorotheophylline were titrated potentiometrically using a Beckman Model A *p*H meter equipped with glass-calomel electrodes. The solvent employed was 1:1 absolute ethanol and water. The titrant was standard sulfuric acid. The volume of standard acid necessary to titrate each salt to the equivalence point calculated from the weight of sample taken and assuming a 1:1 molecular proportion of acid to base was compared to the volume actually required in the titration. In all cases the salts were found to have a 1:1 proportion, and they all showed a strong buffer action at the equivalent point which is typical of a salt of a weak acid and weak base (6). The titration curve of meperidine 8-chlorotheophyllinate is shown in Fig. 1.

Infrared Spectra.—The spectra of the new derivatives along with that of 8-chlorotheophylline were produced by a Baird Associates, Inc., Model B recording infrared spectrophotometer in the Chemistry Department of The Ohio State University. The instrument was equipped with a sodium chloride prism and a Nujol mull of each compound served as the investigated sample.

The infrared spectra of the synthesized compounds were analyzed by comparison with the spectrum of 8-chlorotheophylline (Fig. 2) and using the tables by Randall, *et al.* (7).

The outstanding feature of all of the infrared spectra is the presence of two prominent bands appearing at 5.62 to 5.82μ and 5.84 to 6.04μ. These two peaks are attributed to the cyclic diacylimide structure (—CO—NH—CO—) which is found in the purine structure of 8-chlorotheophylline. In Fig. 3 the presence of the meperidine residue is indicated by ester carbonyl band at 5.75μ. In Fig. 4 the most significant band which substantiates the presence of the levorphan moiety is the phenyl band at 6.25μ. The ketone carbonyl band at 5.80μ in Fig. 5 is good indication of the presence of the metopon residue in the new derivative.

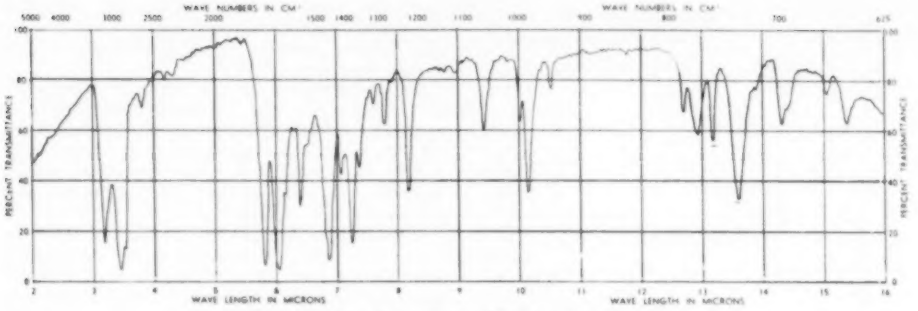

Fig. 2.—Infrared spectrum of 8-chlorotheophylline.

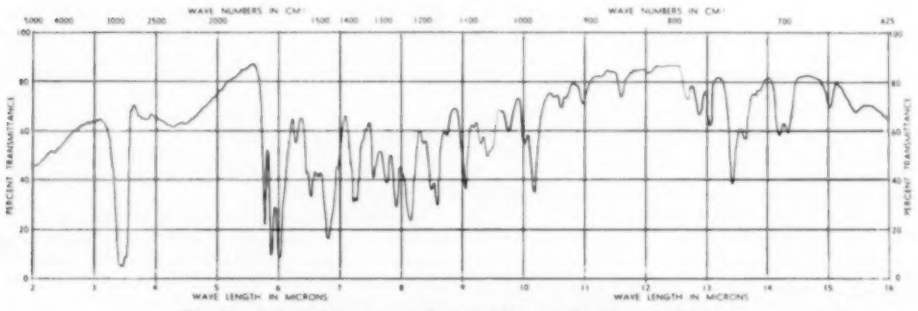

Fig. 3.—Infrared spectrum of meperidine 8-chlorotheophyllinate.

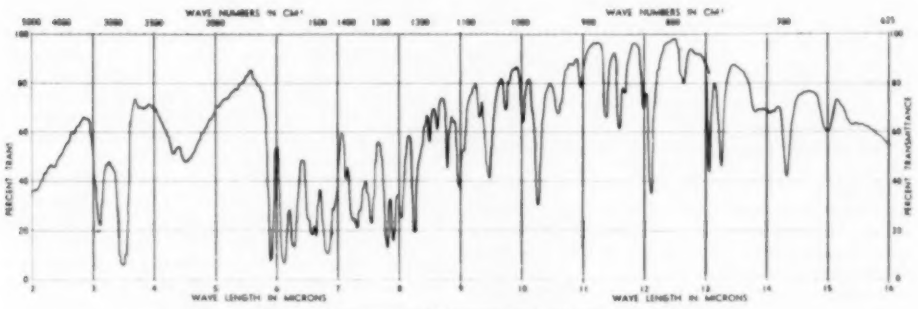

Fig. 4.—Infrared spectrum of levorphan 8-chlorotheophyllinate.

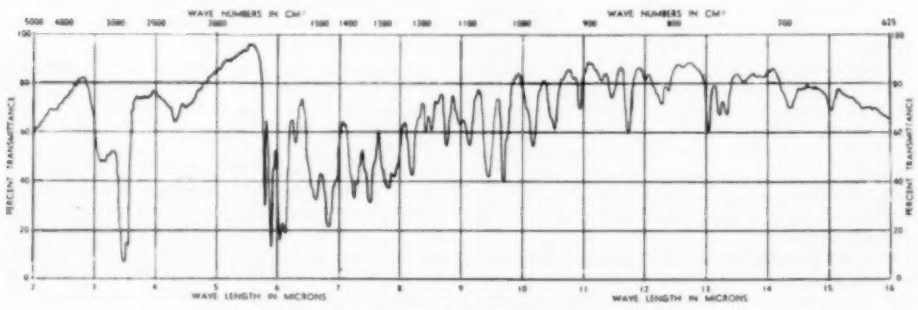

Fig. 5.—Infrared spectrum of metopon 8-chlorotheophyllinate.

SUMMARY

The 8-chlorotheophylline salts of three synthetic narcotic bases including meperidine, levorphan, and metopon were prepared. Some of the optical crystallographic properties of meperidine and levorphan 8-chlorotheophyllinate were studied and the molecular proportion of acid to base in the three new salts was determined by potentiometric titration. The infrared spectra of the compounds were prepared and prominent bands were analyzed.

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The Goldfish as a Potentially Useful Animal for Testing Neurotoxicity of Antibiotics*

By BERTON E. BALLARD†, JEAN DUFRENOY‡, and ROBERTSON PRATT

Goldfish (*Carassius auratus*) exposed to appropriate concentrations of streptomycin evidence difficulty in maintaining equilibrium. Associated with this symptom of toxicity is a pronounced increase in the electrical current required to elicit the normal galvanotropic response. Dihydrostreptomycin does not induce similar effects during exposure of the animals for twenty days, although derangement of equilibrium occurs several months later. Other antibiotics, such as penicillin and Terramycin,[1] do not elicit such responses. The goldfish may be a useful test animal in screening drugs for neurotoxicity, with special reference to the VIII cranial nerve, in man.

FORTUNATELY, most of the common antibiotics do not have serious neurotoxic properties. A notable exception is seen in the treatment of chronic infections with streptomycin or dihydrostreptomycin, neurotoxicity of which in long-term therapy is well known. The literature on the subject has been well reviewed up to 1951 by Berg (1). References (2–9) discuss other work. The cat (10) and other animals have been used in unofficially testing neurotoxicity of streptomycin and its derivatives. The present work was undertaken in an attempt to discover a test animal that could be used for determining neurotoxicity of the streptomycin type, but that would be less expensive than cats or other mammals to acquire and to maintain. Discovery of such a test animal might be an important adjunct for the preclinical testing of new antibiotics and of other drugs. The work reported below suggests that the goldfish may be a satisfactory animal for this purpose.

A secondary scientific benefit that could be derived from more extensive use of fishes in drug studies would be the uncovering of fundamental information concerning this animal group, many members of which are important as food and as sources of drugs and drug products. A case in point is a recent report on the Siamese Fighting Fish as a test animal for the assay of lysergic acid diethylamide (11).

MATERIALS and METHODS

Choice of an Experimental Animal.—Although mammals and birds, in terms of body temperature and homology of tissues and organs, are more closely related to man, the animal chosen for this study was the goldfish, *Carassius auratus*. Several theoretical considerations were involved in the choice. Fish carry on external respiration through their well vascularized gills. Suitable drugs in solution may pass readily across the gill membrane into the blood or body fluids. Thus, drugs that pass through the gill membrane can be administered

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[1] Chas. Pfizer Co.

Thanks are expressed to Dr. John Leighty of the Eli Lilly Company, through whose courtesy the penicillin and streptomycin used in this work were obtained, and to Dr. Gladys Hobby of the Chas. Pfizer Co. for the supplies of dihydrostreptomycin and Terramycin.

easily without injection. If the concentration of drug in the surrounding water is held constant, it might be anticipated that the drug level in the blood and tissues of the fish might also remain relatively constant, after an initial period of adjustment.

Testing Techniques.—On the basis of preliminary trials, two techniques were selected.—*Galvanotropic Response.*—To elicit this response, fish are subjected to direct current provided by suitably setting up electrodes at opposite ends of a test tank. In response to an appropriate current, the fish swim or orient toward the anode. At the minimal effective current, the first evidence of reaction is a twitch. The response is thought to result, in part, from stimulation of the lateral line organ sensorial cells. Thus, measurement of the ability of the fish to respond to the current might be useful in testing neurologic effects of drugs. It was found that goldfish ranging from 3.5 cm. to 5.5 cm. in length (snout to base of caudal fin) respond to a current of about 1.7 milliamperes or more with a 6-volt d. c. source.

"Fin-droop" and Tilt Responses.—These responses can be observed without intricate equipment, and, in the present study, afforded a more practical measure of neurotoxicity. The technique consists simply in observing the animals and noting the number in which the fins, especially the dorsal, are collapsed in contrast to their normal positions; and in measuring the degree of rotation of the animals on their longitudinal axes.

EXPERIMENTAL

The Hypothesis.—It is well known that streptomycin and dihydrostreptomycin have a neurotoxicity for the VIII cranial nerve in man. Streptomycin has its primary toxic action on the nerve fibers in the vestibular branch of the VIII cranial nerve, and secondary action on the cochlear branch. Vertigo is the primary symptom of its intoxication; deafness is a secondary symptom. Dihydrostreptomycin has its primary toxic action on the cochlear branch of the VIII cranial nerve in man, and a secondary action on the vestibular branch. Hearing loss and deafness are the primary symptoms of dihydrostreptomycin toxicity; vertigo is a secondary symptom.

The anatomy of the vestibular cells and of the lateral line sensorial cells of fishes are somewhat similar. However, the functions of the ear and the lateral line organs are not identical, but they both play a part in maintaining the position of fishes in the water (12).

In view of the functional similarity between the organs of equilibrium in man and in fishes, by analogy streptomycin might be expected to impair some of the cells in the vestibule and in the lateral line in fishes. If such impairment did occur, the affected fish would be expected to have difficulty in maintaining balance, and to react to an electrical current less readily than normal fish.

Since dihydrostreptomycin is reported to show little or no vestibular or cochlear damage when used for short-term therapy in man, it would not, by analogy, be expected to cause rapid interference with the lateral line or vestibular organs in fishes. Thus, the normal galvanotropic response would not be expected to be significantly affected in fishes, if they were treated with the drug for short periods.

In man this drug has a primary neurotoxic action on the cochlear branch of the VIII cranial nerve, and often its first neurotoxic symptom is deafness months after therapy has been discontinued: secondarily, dihydrostreptomycin may act on the vestibular branch of the VIII cranial nerve. Since fishes do not have any cochlea, deafness, as we know it, cannot be induced. Yet, in fishes, by analogy, vestibular or lateral line damage should be expected, but the neurotoxic signs should be delayed.

Other antibiotics, such as penicillin, the tetracyclines, etc., would not be expected to affect the normal galvanotropic response and balance of fishes, since these drugs are not reported to exert a neurotoxic action in man.

RESULTS

The Galvanotropic Response.—Twenty-five goldfish were randomly divided into five equal groups and each fish in each group was subjected daily to an electric current to determine the least amperage required to cause it to twitch. When the current was markedly increased above this threshold, the fish swam vigorously toward the anode, irrespective of the direction in which they had been facing before the current was applied.

Before the administration of drug, the normal threshold values were determined by observations made on three consecutive days. Then stock solutions of the antibiotics were added to the tanks in a sufficient volume to give the desired final concentration. To minimize changes in concentration due to metabolic activities of the fish, microbial action, air oxidation, and other factors, the solutions were changed daily. For the actual galvanotropic tests, each animal was removed from the drug-exposure tanks each day, rinsed by passing it through water, and then placed in the electrode-equipped tank.

Data from the galvanotropic experiments are given in Table I. The normal threshold for all fish before the seven-day test period was 1.7 ma. There was a slight increase in the threshold current for all fish, including the controls, during the test period, indicating some decrease in sensitivity to the electrical stimulation. This might be attributed to adaptation. However, it is apparent from the table that the increase in threshold current required for

TABLE I.—INCREASE IN THRESHOLD CURRENT REQUIRED TO INDUCE GALVANOTROPIC RESPONSE IN GOLDFISH EXPOSED TO CERTAIN ANTIBIOTICS (SOURCE—6 VOLTS D. C.)

Drug	Concentration, mg./L.	Increase in Threshold, ma.
Control (H_2O)	...	0.7
Streptomycin SO_4	200	3.6
Dihydrostreptomycin SO_4	170	1.4
Penicillin Na	150	0.7
Terramycin HCl	200	0.6

Fig. 1.—Photographs of a tank of goldfish exposed to streptomycin (200 mg./L.) for ten days. In some instances the same fish can be seen in different views. Left: Light-colored fish in upper left and dark-colored fish in right center show normal fin position and were counted normal. Right: The lighter fish in left center and the darker one below it are examples of fin droop: note especially the positions of the dorsal, caudal, and anal fins.

fish exposed to penicillin or to Terramycin was no greater than for the controls, indicating that those antibiotics had no effect on the galvanotropic response. Streptomycin, on the other hand, caused a marked increase in the amperage required to induce the threshold twitch.

Table I shows that the average rise in current required to elicit the twitch response for five fish exposed to streptomycin was 3.6 ma. However, three of the five fish showed a pronounced increase in threshold, while the other two were less responsive. The average increase in threshold for the three most reactive fish was 4.7 ma.; for the other two animals 1.9 ma. Segregation of a sample consisting of only five fish may seem unwise. However, later experiments with larger populations tested by a different technique indicated that from 50 to 60% of fish were affected by streptomycin.

Fish exposed to dihydrostreptomycin, as would be expected, showed considerably less response than those in streptomycin.

The "Fin Droop" and Tilt Responses.—While collecting data from the galvanotropic tests, it was noticed that the fins of some of the fish exposed to streptomycin tended to lie flat against the body. A typical example of "fin droop" is shown in Fig. 1. Associated with fin droop was a disturbance of equilibrium whether the fish were swimming or not: fish intoxicated with streptomycin tended to roll about the antero-posterior axis. Fin droop was not noted in fish exposed to solutions of dihydrostreptomycin, penicillin, or Terramycin.

The fin droop reponse was reversible. When fish were placed in fresh water after seven days of exposure to streptomycin (200 mg./L.), the fins returned to normal position in a few days.

The first experiment on "fin droop" was to determine (*a*) the percentage of fish that might be expected to evidence this symptom of streptomycin intoxication, and (*b*) the response of goldfish to a second period of exposure.

Initial Intoxication.—Twenty-two fish were placed in a tank and observed for three days while they adapted to their new environment. During this trial all animals appeared normal. There was no evidence of "fin droop." Then streptomycin solution at 200 mg./L. was introduced as described above. The fish were observed daily while they were at rest; for when they were excited and swimming about, the dorsal fin had a tendency to rise momentarily and thus to make the observations less accurate. The results of this experiment are plotted in Fig. 2 (open circles).

It took four days for the first animal to show fin droop, but by the eleventh day 12 fish (55%) showed the symptom clearly. At that time the drug solution was discontinued and all fish were returned to water. After three days in water, all fish had returned to normal. Then the 10 fish which had shown the most severe fin droop were separated from the other animals in the same tank by means of a plastic screen which allowed free diffusion of water and solutes between the portions of the tank now separated. Shortly after this experiment one fish died.

The Follow-up Intoxication.—After the fish had had one week in which to recover from the experiment described above, the 21 survivors were reexposed to streptomycin (200 mg./L.) in the same way as during the first trial. The data, shown in Fig. 2 (solid circles), are substantially similar to those obtained during the initial exposure, except

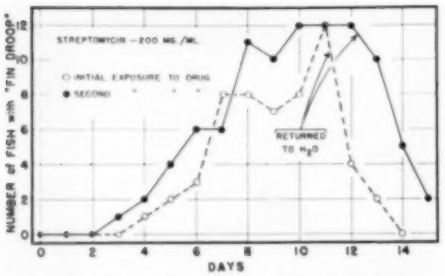

Fig. 2.—Cumulative curve of fish showing fin droop during exposure to streptomycin and their recovery when the drug is withdrawn.

that the onset of reaction to the antibiotic was more rapid. The shape of the retest curve (solid circles) suggests that the fish may have had some residual effects from the first exposure.

It is interesting that of the 10 fish most affected by the drug during the initial exposure nine or 90% of the individuals behind the plastic screen, showed pronounced fin droop in the follow-up exposure. From 40 to 45% of the animals did not show fin droop. The fact that this range was constant in most experiments would have significance if fin droop were used as a test for toxicity.

Nearly all fish tested showed some rotation or rolling about the antero-posterior axis whether or not they exhibited fin droop.

Comparison of Streptomycin with Dihydrostreptomycin.—Thirty-five fish not previously exposed to drugs were used in this test and were divided at random into seven groups of five animals each. The test period was 20 days. Three of the groups were exposed to streptomycin, at concentrations of 100 mg., 200 mg., and 400 mg./L., respectively. Three groups were exposed to dihydrostreptomycin at the same concentrations. One group served as a water control.

Fish exposed to streptomycin showed much the same response as in the previous experiment; 60% of the animals showed marked fin droop. The concentration of antibiotic seemed to have no significant effect on the number of fish responding, suggesting that even the lowest concentration used (100 mg./L.) exceeded the threshold necessary to exert a neurotoxic effect on sensitive fish.

All animals survived during the 20-day test period but during a 22-day recovery period in water following the experiment, two to three (40–60%) of the fish in each tank exposed to streptomycin died. There appears to be a correlation between length of exposure and subsequent mortality rate when the drug is discontinued.

Fish exposed to dihydrostreptomycin, at the several concentrations listed above, did not evidence any visible signs of toxicity during the 20-day test period or during the 22-day recovery observation period in water following exposure to the drug. During the test period one fish died in the 200 mg./L. tank. But this was attributed to natural causes, since no other animals died during either the test period or the follow-up recovery period. Although none of the fish showed symptoms of neurotoxicity during the experiment or during the immediate post-experiment observation period, nearly half of 14 survivors began to swim off keel about five months later, although during this time they were not exposed to drug. The animals appeared to be healthy, except for the fact that they could not properly maintain normal balance. The tilt varied from approximately 5 to 20° off center.

Terramycin was studied at concentrations of 100 mg., 200 mg., and 400 mg. per liter. None of the concentrations of Terramycin elicited any fin droop or imbalance or other evidence of toxicity either during the 20-day test period or during the subsequent observation period of three weeks in water. These negative results are in agreement with those of the earlier test for elevation of galvanotropic threshold, in which Terramycin caused no response.

SUMMARY

1. Goldfish (*Carassius auratus*) placed in an electrical field show a galvanotropic response, i. e., when sufficient current is applied they swim to the anode. At the threshold current required to elicit a response they give a slight twitch. The threshold for goldfish approximately 3.5 to 5.5 cm. long is about 1.7 milliamperes at 6 volts d. c.

2. Exposure of fish to an appropriate concentration of streptomycin markedly increases the threshold current required to elicit galvanotropism. Exposure to an equivalent concentration of dihydrostreptomycin causes only slight or negligible increase. Penicillin and Terramycin at the concentration studied, have no effect on the galvanotropic threshold. It is suggested that measurement of the elevation of galvanotropic threshold current in goldfish may be a useful way of testing drugs preclinically, especially those potentially neurotoxic to the VIII cranial nerve in man.

3. Approximately 60 per cent of goldfish exposed to streptomycin (100 mg. to 400 mg./L.) for a few days evidence fin droop and impairment of equilibrium. Fin droop is a condition in which the dorsal, caudal, and anal fins lie close to the body in contrast to their normal position. Tilt is a condition in which the fish swim and rest off keel. In these experiments fish swam and rested from 5 to 20° off center.

4. Following relatively short exposure (up to twelve days) to streptomycin, fish appear to recover if returned to water not containing the drug. If fish are exposed to the drug for three weeks or more, damage appears to be irreversible.

5. No evidence of fin droop or tilt was seen in fish exposed to dihydrostreptomycin (100 mg. to 400 mg./L.) during twenty days of exposure. However, five months after the drug was dis-

continued and the fish had been returned to antibiotic-free water, about 50 per cent of the animals began to swim off keel. The tilt ranged from 5 to 20° off center.

6. Penicillin and Terramycin did not elicit the fin droop or tilt responses.

7. It is suggested that the manifestation of fin droop or of tilt in goldfish exposed to drugs may be a useful criterion of neurotoxicity.

8. Goldfish give promise of being good test animals for screening water-soluble drugs for neurotoxicity, especially that related to VIII cranial nerve damage in man. Advantages are (*a*) economy in cost and labor of upkeep, (*b*) ease of administration of drug, (*c*) ease of maintaining relatively uniform concentration of drug. The gills seem to be a desirable route for administering water-soluble drugs, since these structures are thin, permeable, and well vascularized.

9. Possible disadvantages of goldfish as test animals: drugs poorly soluble in water could not be so easily administered by the technique employed in the present work; certain drugs might not readily pass through the gill membrane; fishes are not as advanced phylogenetically as mammals and often lack organs, e. g., cochlea, found in higher animals.

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Analysis of Phenobarbital in Pharmaceuticals by Argentimetric Potentiometric Titration*

By JEROME IRWIN BODIN†

Phenobarbital and its sodium salt can be rapidly determined in elixirs, parenteral solutions, capsules and tablets by direct titration in the presence of a variety of standard diluents. Stearates, halides, and ammonium salts interfere and special consideration must be given to samples containing polyethylene glycols, alcohol, or methenamine, if present. A procedure for removal of stearates from tablets is presented.

THIS STUDY was undertaken to determine whether the potentiometric method for phenobarbital elixir described by Mattocks and Voshall (1) could be modified so as to be applicable to the direct titration of phenobarbital in various dosage forms. The potentiometric method is based upon the reaction conditions used by Budde (2) and consists of the quantitative reaction of one mole of silver ion with one mole of barbiturate to form a slightly dissociated salt which is soluble in dilute sodium carbonate solution. At the end point, excess silver ion precipitates silver carbonate such that the end point potential is that of a saturated solution of silver carbonate in the presence of excess carbonate ion.

In a previous paper (3), it was shown that the method as proposed by Mattocks and Voshall was found to be unsatisfactory in that the end point potential of their dead-stop titration was not reproducible. The method described here eliminates the uncertainty in the end point potential by determining the potential of a standard blank solution (saturated with silver carbonate) for each sample just prior to titration. This end point potential varies from time to time depending on the condition of the electrodes, but is relatively constant to ± 1 millivolt for the length of time required to perform several titrations.

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† Fellow of the American Foundation for Pharmaceutical Education, 1954–1955, School of Pharmacy, University of Wisconsin.
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EXPERIMENTAL

Apparatus.—10-ml. buret, Beckman Model G *p*H meter, silver and saturated calomel electrodes, saturated potassium nitrate, agar salt bridge, and mechanical stirrer.

Reagents.—Standard silver nitrate, 0.1 *N*, 0.01 *N* (1:10 dilution); sodium chloride, primary standard; sodium carbonate, A.R., anhydrous, 3% solution; alcohol, 95%; alcohol, absolute; phenobarbital, U. S. P., recrystallized twice from water, m. p. 175–176°.

Preliminary.—The instrument was used on the + m. v. scale. The silver indicator electrode was immersed in the solution to be titrated and a calomel electrode, immersed in a beaker of saturated potassium chloride, was connected to the titration beaker by means of a salt bridge. Solutions were mechanically stirred. The titration curve for 0.1 *N* silver nitrate against 200 mg. phenobarbital in 1.5% sodium carbonate and 10% alcohol solution is shown in Fig. 1(C). Also plotted is the titration curve for 0.01 *N* silver nitrate against a solution blank of the same composition as the sample but with the phenobarbital omitted, Fig. 1(A). A comparison of the two curves shows that the potential equivalent to 1.00 ml. of 0.01 *N* silver nitrate in the blank curve is slightly higher than the inflection point (true equivalence point) in the corresponding phenobarbital curve. This suggested a dead-stop titration of phenobarbital to the potential exhibited by a solvent blank to which was added the equivalent of 0.1 ml. of 0.1 *N* silver nitrate. This volume could then be subtracted from the volume used to titrate the phenobarbital sample.

This method was tested and found to be generally applicable with a few restrictions.

Standardization.—The silver nitrate solution was standardized against primary standard sodium chloride by the conventional potentiometric method. Standardization against recrystallized phenobarbital by the dead-stop procedure gave the same normality, within experimental error.

Standard Blank.—For all titrations, with the exception of the elixir, a solution referred to as the standard blank was freshly prepared. This solution was of the same composition as the solvent in which the sample was dissolved. The solution consisted of 10.00 ml. 95% alcohol, 50 ml. 3% sodium carbonate solution, 1.00 ml. 0.01 *N* silver nitrate, and distilled water to make 100 ml. The potential of the standard blank solution was determined prior to the titration of each sample, and it served as the end point potential to which the sample was titrated. The potential of the standard blank solution was 232 ± 12 millivolts *versus* the saturated calomel electrode. To correct for the blank, 0.1 ml. was subtracted from the volume of 0.1 *N* silver nitrate used for each titration.

Titrations.—*General Procedure. For Bulk Phenobarbital and Phenobarbital Sodium, Tablets and Capsules (Stearates Absent), and Parenteral Solutions.*—Weigh (or measure) a sample equivalent to approximately 200 mg. phenobarbital and mix with exactly 10 ml. 95% alcohol. Add 50 ml. 3% sodium carbonate solution and distilled water to make 100 ml. Titrate to the standard blank potential.

Procedure for Elixir Phenobarbital, U. S. P.—Measure 50 ml. of the elixir and mix with 50 ml. 3% sodium carbonate solution. Prepare a reference blank solution consisting of 7.00 ml. absolute alcohol 50 ml. 3% sodium carbonate solution, 1.00 ml. 0.01 *N* silver nitrate, and distilled water to make 100 ml. Determine the potential of the reference blank solution and titrate the sample to this potential.

Procedure for Tablets and Capsules (Stearates Present.)—Weigh a sample equivalent to approximately 500 mg. phenobarbital and transfer to a 250-ml. volumetric flask with the aid of exactly 25 ml. 95% alcohol. Add 25 ml. distilled water and swirl flask till undissolved solid is evenly dispersed. Add 125 ml. 3% sodium carbonate solution, shake flask thoroughly, and dilute to mark with distilled water. Filter, preferably with suction, through a

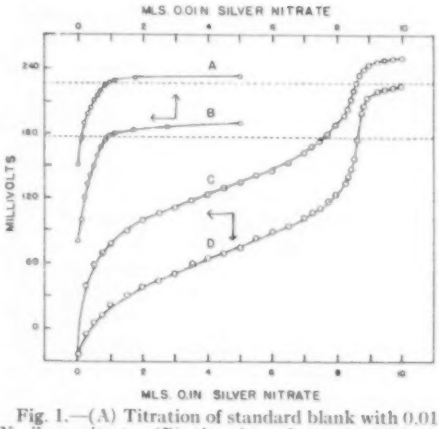

Fig. 1.—(A) Titration of standard blank with 0.01 *N* silver nitrate, (B) titration of standard blank including 25% acetone with 0.01 *N* silver nitrate, (C) titration of 200 mg. phenobarbital in standard blank solvent with 0.1 *N* silver nitrate, (D) titration of 200 mg. phenobarbital in standard blank plus 25% acetone solvent with 0.1 *N* silver nitrate.

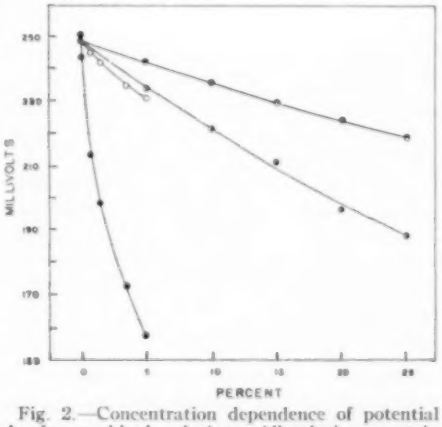

Fig. 2.—Concentration dependence of potential of reference blank solution. All solutions contain 1.5% sodium carbonate solution plus 1.00 ml. 0.01 *N* silver nitrate. ●—● methenamine, ◓—◓ alcohol, ◑—◑ acetone, ○—○ polyethylene glycol 4000.

fine, retentive filter paper,[1] discarding the first 25 ml. Collect the remainder in a dry receiver and titrate 100-ml. aliquots to the standard blank potential.

RESULTS

Applicability and Limitations.—The direct titration method was tested on samples containing 200 mg. phenobarbital in the presence of a selected number of materials likely to be present in any of the dosage forms. The ratio of material to phenobarbital was greater than the maximum commonly encountered in standard dosage forms. Three analyses were made of each type of sample. The results obtained for noninterfering materials are averaged in Table I. Precision is expressed as the average deviation of a single result.

TABLE I.—RECOVERY OF PHENOBARBITAL IN THE PRESENCE OF NONINTERFERING MATERIALS

Material	Amount	Phenobarbital Recovered, %
Starch	1 Gm.	100.0 ± 0.1
Dextrin	1 Gm.	100.0 ± 0.0
Sodium sulfate	1 Gm.	100.2 ± 0.0
Acacia	1 Gm.	100.1 ± 0.2
Methyl cellulose 400	1 Gm.	100.1 ± 0.2
Sodium carboxymethyl-cellulose (medium)	1 Gm.	100.1 ± 0.1
Boric acid	0.1 Gm.	100.0 ± 0.3
Glycerin	10 ml.	100.1 ± 0.1
Propylene glycol	10 ml.	100.2 ± 0.1
Lactose	2 Gm.	100.0 ± 0.1
Sorbitol (Sorbo, 70%)	2 ml.	100.2 ± 0.1
Dextrose	2 Gm.	99.9 ± 0.2
Sucrose	2 Gm.	100.4 ± 0.2

The method was found to be inapplicable in the presence of halides, ammonium ion, and sodium, calcium, and magnesium stearates. It was also observed that the potential of the standard blank was reduced, by an amount varying with the concentration, when polyethylene glycols 4000 or 400, alcohol, methenamine or acetone was added to the blank solution. Plots of the concentration dependency of the end point potential for some of these materials are shown in Fig. 2.

Analysis of Standard Dosage Forms.—Bulk phenobarbital, elixir phenobarbital, U. S. P., and the following tablet formulations were selected as representative products.

Formula I		Formula II	
Phenobarbital	15 mg.	Phenobarbital	30 mg.
Lactose	56 mg.	Sucrose	136 mg.
Sucrose	100 mg.	Dextrin	10 mg.
Starch	20 mg.	Starch	20 mg.
Acacia	5 mg.	Calcium stearate	4 mg.
Magnesium stearate	4 mg.		200 mg.
	200 mg.		

The results of the analyses of these samples are given in Table II. The methods used were those outlined in the Experimental section.

[1] Whatman No. 42 is satisfactory.

TABLE II.—ANALYSIS OF BULK PHENOBARBITAL AND DOSAGE FORMS

	No. Samples	Recovery, %
Bulk phenobarbital	10	100.0 ± 0.2
Elixir phenobarbital	13	99.8 ± 0.2
Tablet Formula I	4	100.2 ± 0.3
Tablet Formula II	7	99.9 ± 0.1

DISCUSSION

The experimental data show that the dead-stop potentiometric titration procedure is applicable to the direct analysis of phenobarbital in the presence of a wide variety of extraneous materials. Since the titration is performed in an alkaline medium, phenobarbital sodium may also be analyzed by this method. The proposed procedure is capable of high accuracy and precision and may be performed rapidly.

Substances which produce a concentration-dependent depression of the end point potential may be present, but their concentration must be known within certain limits. The limits vary from substance to substance, but may be estimated from graphs such as those shown in Fig. 2. The presence of small amounts of such substances may be ignored provided that the difference between the end point potential and that of the standard blank is not greater than two millivolts. Under these conditions, an accuracy of two parts per thousand may be achieved. Thus, the polyethylene glycol 400 in samples of parenteral solutions containing as much as 2 ml. of this substance may be ignored, since the potential depression of the standard blank containing added polyethylene glycol 400 is of the order of one millivolt per milliliter.

Where the presence of such substances cannot be ignored, as in the case of appreciable concentrations of alcohol, the amount present must be estimated and included in the blank. Thus, the average alcohol content of phenobarbital elixir is assumed to be 14% and 7% alcohol is included in the blank since the elixir is diluted in half for titration. No measurable error is introduced in assuming the alcohol content to be 14% provided that the true concentration is 11–17%.

If the concentration of substances such as alcohol or methenamine is not known to a good approximation, ordinary potentiometric titration methods may be employed and the end point determined graphically. The end point may be sharpened by the inclusion of a minimum of 25% acetone. Fig. 1(D) shows the effect of 25% acetone in repressing the dissociation of silver phenobarbital and increasing the slope of the inflection point. The corresponding titration curve for 0.01 *N* silver nitrate against a solvent blank of the same composition is shown in Fig. 1(B). No advantage was observed in the use of excessive quantities of acetone or alcohol by the dead-stop procedure.

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A Note on an Electrolytic Synthesis of I^{131} Labeled Iodoform*

By P. W. GERDING†, J. E. CHRISTIAN, and G. J. SPERANDIO

The procedure described involves a two-step conversion of radio sodium iodide (I^{131}) to iodoform by electrolysis in the presence of acetone, resulting in a yield of 22.8 per cent.

IODINE-131[1] is readily available and inexpensive in the form of sodium iodide (I^{131}). Consequently the synthesis procedure developed was based on the use of sodium iodide as one of the starting materials and involved a two-step electrolytic conversion to iodoform in the presence of acetone, the procedure being a modification of a method described by Teeple (1) in 1904. After electrolysis begins, two reactions occur concurrently and are represented by the following equations:

$$6\ NaI - 6e \rightleftarrows 3\ I_2 + 6\ Na^+$$

$$3\ I_2 + C_2H_6O + H_2O \rightarrow CHI_3 + 3HI + CH_3COOH$$

The most convenient arrangement for carrying out these reactions was to employ the Sargent-Slomin electrolytic analyzer, using a rotating platinum gauze anode to keep the solution in active motion. The area of the anode was 6.5 square inches. The cathode consisted of a platinum wire three inches in length. The apparatus was set up with an ice bath for the electrolysis beaker in a closed hood designed for work with radioactivity. The temperature was not allowed to exceed 20° so that the formation of sodium iodate was kept at a minimum.

Distilled water (112.5 ml.), 12.5 Gm. of sodium iodide, and 1.0 ml. of acetone were placed in an electrolytic beaker. The electrodes were placed into the lower half of the solution and stirring was initiated. Thorough stirring was necessary so that the very dilute solution of iodine produced at the anode could always react with the very dilute solution of sodium hydroxide produced at the cathode.

The current was adjusted to 0.84 amp., starting the electrolysis. A comparatively low anode current density of 0.84 amp./6.5 in.^{2} (2 amp./dm.^{2}) is produced which prevents the formation of iodate and the high cathode current density prevents the reduction of hypoiodite already formed. Approximately 0.80 Gm. of iodine (one-half the amount set free by the electric current) was added in small portions as necessary to keep the solution just slightly colored (acidic), to avoid an excess of alkali.

The problem of foaming which occurs was satisfactorily dealt with by washing down the foam with water from a wash bottle at periodic intervals.

After the reaction had been running for fifteen minutes, 0.1 mc. of NaI^{131} solution was accurately delivered from a micropipet to the reaction mixture along with any remaining portion of the iodine. When a total reaction time of thirty minutes had elapsed, the electrodes were removed from the reaction mixture and any adhering particles washed into the reaction mixture with distilled water. The iodoform was collected by filtration, washed several times with distilled water and dried in a desiccator at room temperature for two days, at which time constant weight was obtained. The iodoform was recrystallized from ethyl alcohol.

Three nonradioactive trial runs were made with yields of 1.28, 1.34, and 1.37 Gm., representing yields of 21.6, 22.8, and 23.2 per cent. Melting points taken on the three separate products obtained were 118.9°, 119.0°, and 119.0° (total immersion), respectively. The literature gives the following melting points for iodoform: 115° (2), 119° (3), and 120° (4).

The yield of a single radioactive run was 1.34 Gm., representing a yield of 22.8 per cent. The iodoform so produced had an approximate specific activity of 4.0 μc./Gm. of material, representing an isotopic yield of 5.4 per cent.

The radiochemical purity of the I^{131} labeled iodoform was established by using ascending paper partition chromatography. The solvent system consisted of 75 ml. methyl alcohol and 25 ml. distilled water. It was demonstrated by examining the paper strips under ultraviolet light that sodium iodide and sodium iodate proceed up the chromatography strip according to their respective R_f values and that iodoform is completely volatilized off the strip after it has traveled two or three inches. By monitoring the strips with a counting rate meter, any radioactive iodide, iodate, and other nonvolatile impurities present could be readily detected. It was found that the radioactive iodoform was free of other iodine-131 containing compounds.

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* Received May 5, 1955, from the Purdue University School of Pharmacy, Lafayette, Ind.
Presented to the Scientific Section, A. Ph. A., Miami Beach meeting, May 1955.
† Present Address, Miles Laboratories, Inc., Elkhart Ind.
[1] The I^{131} used in this research was obtained on allocation by the United States Atomic Energy Commission.

Antibiotics and Thyroid Tissue Slices*

By S. W. MAYER, F. H. KELLY, and M. E. MORTON

In view of the lack of concurrence among several investigators concerning a possible *in vivo* antithyroid effect of Aureomycin® and penicillin, the surviving thyroid slice method in use in this laboratory for several years has been employed to test the direct antithyroid action of some common antibiotics. Although the agents (PAS and isoniazid) frequently employed in treating tuberculosis have been found to exert a significant antithyroid activity, none of the common antibiotics tested exhibited an antithyroid effect except at concentrations higher than those to be expected in clinical practice.

CALESNICK, Harris and Jones (1) recently reported that Aureomycin® and penicillin have an antithyroid effect on rats. Grant (2) and Libby and Meites (3) tested Aureomycin® but could not find an antithyroid effect; Grant reported a "small but definite increase in the uptake of I^{131} by the thyroid gland." We have recently completed a study utilizing the surviving thyroid-slice procedure (4) for investigating the mechanism of the direct antithyroid action of drugs employed in the treatment of tuberculosis. The antibiotics, streptomycin and dihydrostreptomycin, were found to have no direct antithyroid effect, whereas isoniazid and *para*-aminosalicylic acid at concentrations of 10^{-3} molar inhibited thyroidal organic binding of iodine by factors of 2.6 and 16, respectively (5). Inasmuch as extrathyroidal effects may provide the bases for the conflicting reports relative to the action of aureomycin on the thyroid, we have utilized the surviving thyroid-slice procedure to test the direct action of Aureomycin®, penicillin, and several other relatively commonly used antibiotics, and find that they have slight, if any, direct effect on the thyroid—although the concentrations of antibiotics were higher than would be expected in tissues.

EXPERIMENTAL

Previously developed procedures (4, 6) were employed for carrying out surviving thyroid slice incubation with I^{131} as a tracer and subsequent analysis for metabolic iodine products within the thyroid slices. One hundred and fifty milligrams of surviving thyroid slices from freshly slaughtered steers were incubated (Dubnoff Metabolic Shaker) in 3.0 ml. Krebs-Ringer's bicarbonate buffer (*p*H 7.3) for two hours at 37.5°, in the presence of 0.01 microcurie of I^{131} and the tabulated quantity of aureomycin. The supernatant buffer was then decanted, the slices washed with two 1.0-ml. portions of buffer (at 0°) and the total I^{131} content of the slices was measured. Protein-bound I^{131}, tyrosine I^{131}, and thyronine I^{131} fractions in the slices were also determined (4). A well-type scintillation counter was employed for measuring the I^{131} content of the fractions. The incubations were carried out in quadruplicate.

TABLE I.—DISTRIBUTION OF RADIO-IODINE IN SURVIVING THYROID SLICES

Compound	Mg. of Compound per 3.00 ml. of Incubation Medium	Total I^{131} Slices	Per Cent of Initial I^{131} Protein Bound I^{131}	Inorganic I^{131}	Iodinated Tyrosine I^{131}	Thyroxine Thyronines I^{131}
Control	0	63.6 ± 3.8	50.8 ± 3.6	12.6 ± 0.8	44.2 ± 3.3	6.7 ± 0.7
Aureomycin®	10	61.3 ± 4.0	49.3 ± 3.9	12.0 ± 1.1	42.7 ± 3.3	6.5 ± 0.8
Aureomycin®	1.0	70.1 ± 3.7	55.3 ± 3.5	15.1 ± 1.0	47.6 ± 2.9	7.3 ± 0.8
K Penicillin G	$10^{[a]}$	39.7 ± 3.8	29.5 ± 2.8	9.8 ± 0.9	25.7 ± 2.3	3.7 ± 0.3
K Penicillin G	$10^{[a]}$	56.3 ± 3.8	43.2 ± 2.7	12.4 ± 0.5	37.6 ± 2.0	5.4 ± 0.3
K Penicillin G	$10^{[a]}$	64.8 ± 1.3	50.3 ± 1.3	14.0 ± 0.5	46.2 ± 1.1	6.0 ± 0.3
Oxytetracycline	10	45.2 ± 1.9	29.0 ± 1.5	15.9 ± 0.3	25.5 ± 1.6	3.7 ± 0.2
Oxytetracycline	1.0	63.7 ± 0.8	48.8 ± 0.5	14.0 ± 0.5	43.1 ± 1.1	5.8 ± 0.4
Tetracycline	10	48.2 ± 3.9	35.8 ± 3.3	12.0 ± 1.0	31.3 ± 3.0	4.5 ± 0.4
Tetracycline	1.0	62.7 ± 3.1	49.4 ± 3.0	12.8 ± 0.5	43.5 ± 3.1	5.9 ± 0.5
Erythromycin	10	53.1 ± 2.5	42.2 ± 2.4	9.8 ± 0.7	37.1 ± 2.7	5.2 ± 0.4
Erythromycin	1.0	61.8 ± 5.3	46.3 ± 4.1	15.1 ± 4.0	41.1 ± 4.0	5.5 ± 0.3
Thiouracil	0.38	24.1 ± 2.2	2.7 ± 0.2	21.0 ± 2.1	2.3 ± 0.1	0.4 ± 0.1
Thiouracil	0.038	25.2 ± 2.0	6.0 ± 0.4	19.3 ± 1.6	5.3 ± 0.4	0.5 ± 0.1

[a] Quantities of penicillin are expressed in units, rather than milligrams.

RESULTS

In Table I the results are summarized along with

* Received June 26, 1955, from the Radioisotope Research Unit, Investigative Medicine Service, Veterans Administration Hospital, Long Beach, California, and the School of Medicine, University of California at Los Angeles.

This work was supported in part by Grant A-798 from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

the corresponding data for thiouracil, which has a somewhat weaker antithyroid action than the antithyroid compounds currently in clinical use (7). Since the inorganic and tyrosine iodine fractions of the thyroid have relatively negligible hormonal effects, the protein-bound and thyroxine (including triiodothyronine) iodine fractions are taken as a better measure of thyroid hormone synthesis (6, 8). On that basis, there was a statistically significant (at approximately the 95% confidence level) antithyroid effect for only the highest tested concentrations of penicillin, oxytetracycline, tetracycline, and erythromycin. These highest concentrations correspond, for a 60-kilogram man, to a retained dose of 2×10^9 units of penicillin or 200 grams of the other antibiotics—doses very much higher than those actually administered for treatment. Moreover, thiouracil produced a considerably stronger direct antithyroid effect, at a much lower concentration. Although Aureomycin® at 0.33 mg./ml. produced a protein-bound I^{131} level higher than that of the controls, the increase was not statistically reliable.

SUMMARY

The tested antibiotics had no direct antithyroid action, except at much higher concentrations than those normally encountered.

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Book Notices

The Pharmacist in Retail Distribution. By A. HAMILTON CHUTE and ESTER J. W. HALL. Hemphill's, Austin, Texas, 1955. viii + 326 pp. 18 x 26 cm.

This is a revised edition of the 1953 text which was partly a revision of *Retailing by Pharmacists* by A. Hamilton Chute, published in 1941. Changes in this revision have not been sufficient to outmode the 1953 printing substantially. Illustrations and citations have been modernized where feasible. Some material has been added in connection with public relations, the background of marketing by manufacturers, the use of financial ratios for control, and store layout for self-service operation.

The text consists of twenty-six chapters covering all phases of the establishment and operation of a retail pharmacy. It should prove to be extremely useful in the teaching of courses in drug store or pharmacy management. The arrangement of material conforms largely to the outline recommended by a committee as reported in *The Pharmaceutical Curriculum*. Although many important subjects within the text are given limited treatment, this is to be expected in a text intended for use in a course such as drug store management which covers a wide range of subject matter, much of which constitutes separate courses within the curricula of schools of business and law.

The many footnotes used throughout the text reveal the closeness of the authors to current thinking in connection with the writings of other experts in the various phases of drug store operations and also with what is being said at national and state conventions of pharmaceutical organizations and at refresher and seminar-type meetings.

While the text was originally intended for students enrolled in colleges of pharmacy, it should also be found useful by the practicing pharmacist.

Recent Progress in Hormone Research. Vol. 11. The proceedings of the Laurentian Hormone Conference. GREGORY PINCUS, Editor. Academic Press, Inc., New York, 1955. 518 pp. 16 x 23.5 cm. Price $10.

The papers included in this symposium on hormone research are grouped under the headings: pituitary hormones, steroid chemistry and biochemistry, hormones and abnormal growth, hormones and aging in man, mechanism of hormone action, and hormone-cardiovascular interrelations. Particularly interesting papers discuss the use of microorganisms in the synthesis of steroid hormones; the isolation, chemistry, and physiology of aldosterone, and effects of ions and hormones on carbohydrate

metabolism. Each paper and its reference list is followed by the recorded discussion which brings out additional valuable information.

Author and subject indexes for Vol. XI and a cumulative subject index for Vols. I–X are appended. The book is a must for advanced workers in this field.

Medical Research—A Midcentury Survey. Vol. 1. American Medical Research in Principles and Practice. xxxii + 765 pp. 16.5 x 23.5 cm. Vol. 2. Unsolved Clinical Problems in Biological Perspective. xxxii + 740 pp. 16.5 x 23.5 cm. By ESTHER EVERETT and ASSOCIATES. Published for The American Foundation by Little, Brown and Company, Boston, 1955. Price (2 vols.) $15.

"In all of these studies," says the chairman, "our objective has been to set up defenses against the lack of fact and the weight of particular propaganda that inhibit clear thinking on many important questions."

The American Foundation has done a splendid job with this objective in presenting a survey of current medical research. "Current" in these volumes connotes the period from 1940 to 1955. Their emphasis is on the biological approach and the applications of chemistry, physics, and mathematics to biology. Their thesis is that the solution of all clinical problems involves *fundamental* biological research. Therefore, a survey was made of the various agencies where such research was in progress; medical schools, university departments, foundations, private research institutes, hospitals and clinics, industrial organizations, government agencies, scientific and professional societies, and of course, the National Academy of Sciences. The current general trends and problems are discussed as well as many particular schools and institutions. As research cannot proceed without money, the financial picture is also presented, in general and in considerable detail. For instance, it has been estimated that the pharmaceutical industry spends $100,000,000 a year for research.

Volume II on unsolved clinical problems contains ten chapters: Current Metabolic Concepts; Cancer; Infertility; Arteriosclerosis; Hypertension; The Rheumatic Syndromes; Tuberculosis; The Nature of Virus Diseases; Alcoholism; and Biology of Schizophrenia.

"The dominant purpose in each of these chapters is the same—to present the areas of research, however vast, however remote, however little developed as yet, that seem to bear upon the metabolic mechanism primarily involved in the given condition."

This study must have been hampered by the plethora of papers that are ground out each year by investigators who are more concerned with having a long list of publications with which to impress universities than with waiting until they have a worth-while monograph to contribute. It is not always easy to judge what may be an important contribution but there are thousands of papers that serve merely to clog the wheels of progress.

Names and years appear throughout the text of necessity although these are played down and the cooperative and cumulative effort stressed. The authors quote—"Paul plants and Apollos waters and God gives the increase—frequently to neither Paul nor Apollos."

The facts are so interesting and the books so well written that the layman will enjoy them almost as much as those in all branches of medical research.

Major sources are appended to each volume. There is no index but after each chapter heading in the body of the book is a detailed table of contents of that chapter. The type is large.

Public Relations for the Pharmacist. By WILLIAM H. HULL. J. B. Lippincott Company, Philadelphia, 1955. xxi + 132 pp. 16 x 23.5 cm. Price $4.50.

In this new book the author is "... attempting to emphasize the pharmacist's role as an assistant to the physician and as a friend to the patient, with the end result being favored by both the physician and the patient toward the pharmacist. In other words: public relations becomes any action to influence physicians and the public favorably in the pharmacist's behalf."

The phrase public relations has so many different meanings that it is often difficult to understand just what is meant. In defining public relations as discussed within his book, the author avoids much of the confusion that has been associated with public relations in general.

The book consists of six sections treating the topics of: Advancing the Pharmacist's Professional Personality; Building Prescription Practice; Pharmacies in Small Towns and Suburbs; The Technical Aspects of Direct Mail; The Pharmacist and Advertising; and Two of Pharmacy's Problems (Dispensing and Keeping Your Self-Respect). A seventh section is devoted to a series of questions which the pharmacist can ask himself in order to gain new insights into his need for a concerted public relations program.

About half the book is devoted to the chapter entitled "Building Prescription Practice." Particularly impressive in this section are the illustrations and reproductions of letters and other promotional pieces used by successful pharmacists in all parts of the nation in their contacts with physicians and with the public at large. Actually, the book might have more correctly been given the title, "Building Prescription Practice."

The book has much in its favor in view of its brevity and the avowed lack of time of most pharmacists for doing outside reading. The concise, easy-to-read style lends itself to frequent reviews by pharmacists in determining whether or not they have been utilizing all available promotional methods in developing and strengthening their professional reputations.

Even though the average experienced pharmacist is familiar with much of the material presented in this reference, he can still secure an extraordinary dividend on his investment if he derives but one successful idea from the author's presentation on how to develop more prescription volume and maintain a high level of professional respectability in so doing.

Remington's Practice of Pharmacy. 11th ed. ERIC W. MARTIN and E. FULLERTON COOK, Editors. Mack Publishing Company, Easton, Pa., 1956. xii + 1707 pp. 6.5 x 22.5 x 29.5 cm. Price $18.

The rapid pace of developments in pharmacy and its allied sciences is clearly reflected in the thorough revision of this book. However, the complete coverage of this volume is indicated by its thorough treatment of what is important to pharmacy whether it is old or new; historical backgrounds to newest drugs and applications of statistical methods to analytical pharmacy.

The grand scale of this treatise on pharmacy has still retained the admirable simplicity and clarity of presentation of elementary and advanced subject matter that users of earlier editions of *Remington's Practice of Pharmacy* have found so satisfactory. Judicious use of space and deletion of expendable material have permitted inclusion of much valuable information with an increase of only ninety-one pages over the 1616 pages of R. P. P. X. All text material relating to standard references has been brought into conformity with U. S. P. XV, N. F. X, B. P. 1953, Ph. I. vol. I and II, N. N. R. 1955, etc. Each biological, chemical, pharmacological, and technical division of the pharmaceutical sciences has been thoroughly yet concisely covered by authorities in each field. Editors, Eric W. Martin and E. Fullerton Cook, and associate editors, E. E. Leuallen, Arthur Osol, Linwood F. Tice, and C. T. Van Meter, have enlisted the aid of more than 200 individuals in modernizing the material and its presentation.

An enlarged section on physical pharmacy describes the theory and application of modern techniques such as chromatography and rheology. In the sections devoted to inorganic pharmacy, organic pharmacy, biological pharmacy, and analytical pharmacy, the text includes descriptions, chemistry, methods of preparation, pharmacology, therapeutic and pharmaceutical uses for official and important unofficial products such as vaccines (including poliomyelitis vaccine), diagnostic agents, vitamins, and other natural and synthetic compounds. Antibiotics, antihistamines, hormones (including prednisolone and prednisone), are fully covered. In any modern compilation of drug products, the rapidly changing picture makes revision a continuous necessity and it is practically impossible to keep informed of all the changes made. It is no reflection on the editors, who have made an excellent tabulation of antihistamines and products including these drugs, that Harv-amine (Harvey), containing pyrilamine maleate, page 859, is now Harvamine (Harvey), containing phenyltoloxamine dihydrogen citrate.

Pharmaceutical dosage forms are covered in detail with extensive sections on manufacturing of tablets and other products and with special emphasis given to parenteral preparations, ophthalmic solutions, and ointments. The useful information on specialties, including their composition, uses, and doses, includes old and new unofficial products.

Hospital pharmacy is discussed at length, bringing together in a drastically revised chapter much information in this important and rapidly developing field. The names of Don E. Francke and Herbert L. Flack among the assistant editors is assurance that this material has been handled realistically by practicing hospital pharmacists.

The usual readable style, type and format of *Remington's Practice of Pharmacy* is continued in this edition. Use is made of the inside covers with heavy paste-in speets carrying tables of metric doses with approximate apothecary equivalents and logarithms (front) and a periodic chart of the elements (back). The text is replete with illustrations and includes convenient tabulations of surface-active agents, antihistamines, antibiotics, etc. The extensive index is necessary to the convenient use of the wealth of material in the text.

The editors and their associates are to be congratulated on this excellent revision of a book that should be available to all who are interested in the study and practice of pharmacy and its allied sciences.

Resonance in Organic Chemistry. By GEORGE WILLARD WHELAND. John Wiley & Sons, Inc., New York, 1955. xiii + 846 pp. 16 x 23.5 cm. Price $15.

This book is a revised and expanded successor to the author's "*The Theory of Resonance and Its Application to Organic Chemistry.*" An indication of the growth of the subject in the ten years between publications is the Appendix which consists of a table of bond lengths and bond angles and which has expanded from 11 pages to 105 pages. The book differs from the conventional treatment of organic chemistry in its explicit concern with the application of quantum mechanical methods to problems of molecular structure. Its treatment of the subject is sound but can be followed without any special background in quantum mechanics. Except for the final chapter, the treatment is qualitative and nonmathematical. The molecular-orbital method for treating molecular structure is discussed in some detail. The mathematical background of the resonance concept is discussed in the newly added final chapter. The text material is divided under the following chapter headings: The Theory of Resonance, The Nature of Valence, Resonance Energy, Steric Effects of Resonance, Resonance and Dipole Moments, Resonance and Molecular Spectra, Resonance and Chemical Equilibrium, Resonance and Chemical Reaction, Mathematical Basis of Resonance. The 531 references cited in the text are listed at the end and author and subject indexes are included. The author feels that this new presentation of his material is significantly clearer and less confusing than the original publication. The format and printing of the book are good, the molecular and mathematical formulas being easily read. For anyone interested in this field the book is a must.

The Preservation of Natural History Specimens. Vol. 1. Invertebrates. By REGINALD WAGSTAFFE and J. HAVELOCK FIDLER. The Philosophical Library, Inc., New York, 1955. xiii + 205 pp. 19.5 x 25.5 cm. Price $10.

This volume brings together accepted methods which enable demonstration and permanent preservation of taxonomic characters of specimens from most groups of invertebrates. The methods are clearly and completely described.

The Genus Nicotiana. By THOMAS HARPER GOODSPEED. Chronica Botanica Company, Waltham, Mass., 1954, and Stechert-Hafner, Inc., New York, 1955. Illustrated. xxii + 536 pp. 16.5 x 25 cm. Price $12.50.

Parts I to IV of *The Genus Nicotiana* document an interpretation (Part V) of the origins, relationships and evolution of the species of a representative genus of flowering plants based upon accumulated evidence of their distribution, morphology and cytogenetics. Present distribution of all species of the genus, mapped and interpreted in the light of the geologic history of the Americas, Antarctica, and Australasia, is shown to correlate with other fundamental evidence of their phylesis. A living collection of fifty-six of the sixty recognized species and of numerous varieties and forms has permitted comprehensive studies of comparative morphology and cytology which have contributed significantly to documentation of the author's phyletic conclusions.

Amplifying discussions of comparative morphology are full page illustrations representing distinctions in leaf type, inflorescence types and their derivation, corolla parts, aestivation, stamen insertion, anther, stigma, ovary, capsule, and seed morphology and the various trichome types. In the section on cytology karyotypes of all but four species and complete meiotic sequences in representative species and hybrids are illustrated. The living collection has also made possible extensive hybridization involving almost every species. Meiotic chromosome behavior in over two hundred F_1 interspecific hybrids, representing intersectional or intersubgeneric, as well as intrasectional combinations, has been analyzed and tabulated and in many cases illustrated. The combined evidence points to evolution of the genus via amphiploidy superimposed upon amphiploidy with introgressive hybridization and chromosome reorganization contributing largely to genetic differentiation on each successive amphiploid level.

The complete evolutionary picture is presented in charts showing the derivation of each of the sixty species. Part VI (with H. M. Wheeler and P. C. Hutchison, Sr., Botanists, Botanical Garden, University of California, Berkeley) is a taxonomic reorganization of the genus reflecting the relationships demonstrated in Part V. There are keys to all subgeneric categories, descriptions of all categories, including several new sections, varieties and combinations, and full page illustrations of species. The book was published as *Chronica Botanica, Volume 16*.

The Quantitative Analysis of Drugs. By D. C. GARRATT. Philosophical Libraries, Inc., New York, 1955. xv + 670 pp. 15 x 23 cm. Price $17.50.

This book is the second edition; the first edition was entitled "Drugs and Galenicals: Their Quantitative Analysis." The author, Chief Analyst for Boots Pure Drug Co. Ltd., has expanded his earlier excellent text by inclusion of new methods and techniques of analyses and their applications, and he has added many new drug monographs. New material that appeared in the 1954 British Pharmaceutical Codex was added to the already prepared text as a supplement. A separate eight-page chapter on physical methods and short notes on other special techniques are appended.

The special value of the text material stems mainly from the author's personal knowledge of and experience with most of the methods included. Although the latest standard American drug compendia referred to are U. S. P. XIV and N. F. IX, the book contains much sound information and should be available to students and practicing analysts of drugs and drug products.

BOOKS RECEIVED

Klinik und Therapie der Vergiftungen. By SVEN MOESCHLIN. Georg Thieme Verlag, Stuttgart, 1956. Intercontinental Medical Book Corporation, New York. xi + 521 pp. 18.5 x 25 cm. Price DM 59. $14.05.

The American Drug Index. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Company, Philadelphia, 1956. 576 pp. 14.5 x 21 cm. Price $5.

Guide to Earning a Living. By ED CUNNINGHAM and LEONARD REED. Simon and Schuster, New York, 1955. 117 pp. 22 x 28.5 cm. Price $3.95.

Resonance in Organic Chemistry. By GEORGE WILLARD WHELAND. John Wiley & Sons, Inc., New York, 1955. xiii + 846 pp. 16 x 23.5 cm. Price $15.

Medical Research—A Midcentury Survey. Vol. 1. American Medical Research in Principle and Practice. xxxii + 765 pp. 16.5 x 23.5 cm. Vol. 2. Unsolved Clinical Problems in Biological Perspective. By ESTHER EVERETT LAPE and ASSOCIATES. Published for The American Foundation by Little, Brown and Company, Boston, 1955. xxxii + 740 pp. 16.5 x 23.5 cm. Price (2 vols.) $15.

Public Relations for the Pharmacist. By WILLIAM H. HULL. J. B. Lippincott Company, Philadelphia, 1955. xii + 132 pp. 16 x 23.5 cm. Price $4.50.

Proposed International Non-Proprietary Names—List IV

In accordance with paragraph 3 of the Procedure for the Selection of Recommended International Non-Proprietary Names for Pharmaceutical Preparations,[1] notice is hereby given that the following names are under consideration by the World Health Organization as proposed international non-proprietary names. Comments on, or formal objections to, proposed names may be forwarded by any person to the Director-General, World Health Organization, Palais des Nations, Geneva, Switzerland, within four months of the date, January, 1956, of their publication in the *Chronicle of the World Health Organization*. The inclusion of a name in this list does not imply any recommendation for the use of the substance in medicine or pharmacy.

Proposed International Non-Proprietary Name (Latin, English)	Chemical Name or Description
acetarsolum acetarsol	3-acetamido-4-hydroxyphenylarsonic acid
acetylcholini chloridum acetylcholine chloride	2-acetoxyethyltrimethylammonium chloride
acidum ascorbicum ascorbic acid	3-oxo-L-gulofuranolactone (enolic form)
acidum folicum folic acid	N-[4′-([[2-amino-4-hydroxy-6-pteridyl]-methyl]amino)benzoyl]-L(+)-glutamic acid
acidum iophenoicum iophenoic acid	α-(3-hydroxy-2,4,6-triiodobenzyl)-butyric acid
acidum nicotinicum nicotinic acid	pyridine-3-carboxylic acid
adipiodonum adipiodone	adipic acid bis-(2,4,6-triiodo-3-carboxyanilide)
aethisteronum ethisterone	17α-ethynyl-17β-hydroxy-3-oxoandrostene-4
aethylis biscoumacetas ethyl biscoumacetate	ethyl 4,4′-dihydroxy-3,3′-dicoumarinylacetate
amidopyrinum amidopyrine	2,3-dimethyl-4-dimethylamino-1-phenyl-5-pyrazolone
aminitrozolum aminitrozole	2-acetamido-5-nitrothiazole
aminophyllinum aminophylline	mixture of theophylline and ethylenediamine
aminopterinum natricum aminopterin sodium	sodium 4-aminofolate
amphetaminum amphetamine	(±) 2-amino-1-phenylpropane
androstanolonum androstanolone	3(α)-hydroxy-17-oxoandrostane
arsthinolum arsthinol	2-(3′-acetamido-4′-hydroxyphenyl)-1,3-dithia-2-arsa-4-cyclopentylmethanol
atropini methonitras atropine methonitrate	(±) 8-methyl-3-tropoyloxytropane nitrate
barbitalum barbital	5,5-diethylbarbituric acid
barbitalum natricum barbital sodium	sodium salt of 5,5-diethylbarbituric acid
benzatropini methanesulfonas benzatropine methanesulfonate	tropine benzhydryl ether methanesulfonate
bromazinum bromazine	2-(4-bromophenyl-phenylmethoxy)-ethyldimethylamine
buclizinum buclizine	1-(4-chlorobenzhydryl)-4-(4-*tert*butylbenzyl)-piperazine
butacainum butacaine	3-dibutylaminopropyl *p*-aminobenzoate
butalbitalum butalbital	5-allyl-5-isobutylbarbituric acid
calciferolum calciferol	
calcii saccharas calcium saccharate	calcium D-glucarate
carbacholum carbachol	carbamoylcholine chloride
carbarsonum carbarsone	4-ureidophenylarsonic acid
carbimazolum carbimazole	2-ethoxycarbonylthio-1-methylimidazole
carbinoxaminum carbinoxamine	2-dimethylaminoethoxy-2-pyridyl-4-chlorophenylmethane
carzenidum carzenide	4-carboxybenzenesulfonamide
cetomacrogolum 1000 cetomacrogol 1000	polyethylene glycol 1000 monocetyl ether
chiniofonum chiniofon	mixture of four parts by weight of 7-iodo-8-hydroxyquinoline-5-sulfonic acid and one part of sodium bicarbonate
chlorbetamidum chlorbetamide	N-(2,4-dichlorobenzyl)-N-(dichloroacetyl)-ethanolamine
chlormerodrinum chlormerodrine	(3-chloromercuri-2-methoxypropyl)-urea
chlorobutanolum chlorobutanol	1,1,1-trichloro-2-methylpropanol-2
chlorocresolum chlorocresol	6-chloro-3-hydroxytoluene
chloroquinum chloroquin	7-chloro-4-(4′-diethylamino-1′-methylbutylamino)-quinoline
chlorphenaminum chlorphenamine	1-(*p*-chlorophenyl)-1-(2-pyridyl)-3-dimethylaminopropane
chlortetracyclinum chlortetracycline	10-chloro-1-dimethylamino-1,4,6,11,12,13,14,18-octahydro-2,5,7,11,14-pentahydroxy-4,6-dioxo-11-methylnaphthacene-3-carbonamide
cholini chloridum choline chloride	2-hydroxyethyltrimethylammonium chloride
conessinum conessine	an alkaloid obtained from the seeds of *Holarrhena antidysenterica* L.

Off. Rec. Wld. Hlth. Org., **60**, 3 (resolution EB 15.R7).

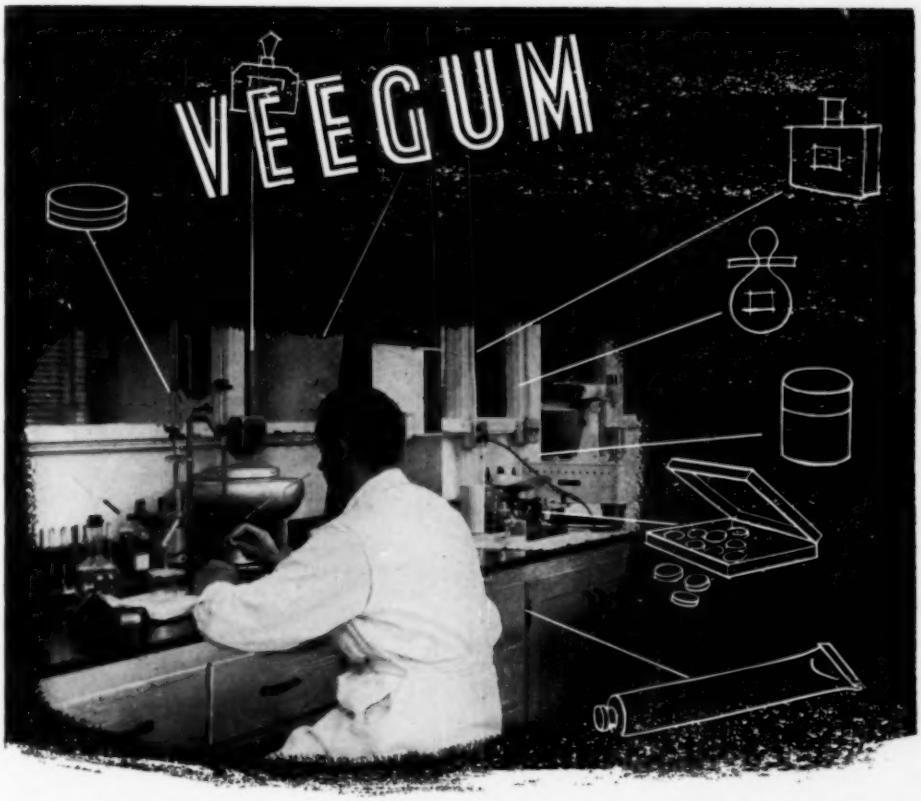
(Proposed International Non-Proprietary Names *contd.*)

cycriminum cycrimine	1-phenyl-1-cyclopentyl-3-piperidino-1-propanol
desoxycortonum desoxycortone	21-hydroxy-3,20-dioxo-pregnene-4
dibrompropamidinum dibrompropamidine	1,3-bis-(4-amidino-2-bromo-phenoxy)-propane
dichloroxylenolum dichloroxylenol	2,4-dichloro-3,5-dimethyl-phenol
diethylstilboestrolum diethylstilboestrol	*trans*-3,4-bis-(4-hydroxy-phenyl)-hexene-3
digoxinum digoxin	glycoside obtained from the leaves of *Digitalis lanata* Ehrh.
dihexyverinum dihexyverine	2-piperidinoethyl 1-cyclo-hexylcyclohexanecarbox-ylate
dihydralazinum dihydralazine	1,4-dihydrazinophthalazine
dimazolum dimazole	2-dimethylamino-6-(2-di-ethylaminoethoxy)-benzo-thiazole
diphemanili methylsul-fas diphemanil methylsul-fate	N,N-dimethyl-4-piperidyli-dene-diphenylmethane methylsulfate
dithranolum dithranol	1,8,9-anthratriol
ectylurea ectylurea	2-ethylcrotonylurea
edrophonii chloridum edrophonium chloride	ethyldimethyl-3-hydroxy-phenylammonium chloride
ergometrinum ergometrine	an alkaloid obtained from ergot
ergotaminum ergotamine	an alkaloid obtained from ergot
erythromycinum erythromycin	an antibiotic produced by a strain of *Streptomyces erythreus*
ethaverinum ethaverine	6,7-diethoxy-1-(3′,4′-di-ethoxybenzyl)-isoquino-line
gitalinum amorphum gitalin amorphous	a glycosidal principle of *Digitalis purpurea* L.
glycerolum glycerol	propanetriol
heparinum heparin	sodium salt of a complex organic acid having the property of delaying the clotting of blood
hexylcainum hexylcaine	1-cyclohexylamino-2-propyl benzoate
hydroxystilbamidinum hydroxystilbamidine	1-(4-amidino-2-hydroxy-phenyl)-2-(4-amidino-phenyl)-ethene
isophanum insulinum isophane insulin	a sterile suspension of insulin with the isophanic equiva-lent of protamine and zinc chloride
lanatosidum C lanatoside C	glycoside obtained from the leaves of *Digitalis lanata* Ehrh.
laudexii methylsulfas laudexium methylsulfate	decamethylene-ω-bis-[1-(3′,4′-dimethoxybenzyl)-1,2,3,4-tetrahydro-6,7-dimethoxy-2,2-dimethyl-isoquinolinium sulfate]
levorphanolum[1] levorphanol	(−) 3-hydroxy-N-methyl-morphinan
levothyroxinum natri-cum levothyroxine sodium	L-β-[(4-hydroxy-3,5-diiodo-phenoxy)-3,5-diiodo-phenyl]-alanine
lobelinum lobeline	L-2-(2-hydroxy-2-phenyl-ethyl)-1-methyl-6-phen-acylpiperidine
lucanthonum lucanthone	1-(2-diethylaminoethyl-amino)-4-methylthiaxan-thone
macrogolum 400 macrogol 400	polyethylene glycol 400
macrogolum 1000 macrogol 1000	polyethylene glycol 1000
macrogolum 4000 macrogol 4000	polyethylene glycol 4000
macrogoli lauras 600 macrogol laurate 600	mono ester of lauric acid and polyethylene glycol 600
macrogoli oleas 600 macrogol oleate 600	mono ester of oleic acid and polyethylene glycol 600
macrogoli stearas 600 macrogol stearate 600	mono ester of stearic acid and polyethylene glycol 600
macrogoli stearas 1000 macrogol stearate 1000	mono ester of stearic acid and polyethylene glycol 1000
meclozinum meclozine	1-(4-chlorobenzhydryl)-4-(3-methylbenzyl)-piperazine
melarsoprolum melarsoprol	2-[4-(4,6-diamino-2-*s*-tri-azinylamino)-phenyl]-4-hydroxymethyl-1,3,2-di-thiaarsenolidine
mepacrinum mepacrine	3-chloro-9-(4′-diethylamino-1′-methylbutylamino)-7-methoxyacridine
meprylcainum meprylcaine	2-methyl-2-propylamino-propyl benzoate
mercumatilinum natri-cum mercumatilin sodium	sodium 8-(2′-methoxy-3′-hy droxy-mercuripropyl)-coumarin-3-carboxylate (sodium mercumallylate) and theophylline
mersalylum mersalyl	sodium salt of 2-[(3-hy-droxymercuri-2-methoxy-propyl)-carbamoyl]-phenoxyacetic acid
methioninum methionine	(±) 2-amino-4-methylthio-butyric acid
methoxaminum methoxamine	2-amino-1-(2,5-dimethoxy-phenyl)-propanol-1
methylcellulosum methylcellulose	cellulose methyl ether con-taining about 30 per cent w/w of methoxyl
methylpentynolum methylpentynol	3-methylpentyn-1-ol-3
methyltestosteronum methyltestosterone	17β-hydroxy-17α-methyl-3-oxoandrostene-4
natrii acetrizoas sodium acetrizoate	sodium 3-acetamido-2,4,6-triiodobenzoate
natrii amidotrizoas sodium amidotrizoate	sodium 3,5-diacetamido-2,4,6-triiodobenzoate
neoarsphenaminum neoarsphenamine	sodium 3,3′-diamino-4,4′-dihydroxyarsenobenzene-N-methylene-sulfoxylate
neostigmini bromidum neostigmine bromide	dimethylcarbamic ester of 3-hydroxyphenyltrimethyl-ammonium bromide
nicethamidum nikethamide	pyridine-3-carboxylic acid diethylamide
nicotinamidum nicotinamide	pyridine-3-carboxylic acid amide
nortestosteroni cypionas nortestosterone cypion-ate	17β-(3-cyclopentylpropio-noxy)-3-oxoestrene-4
oestradioli benzoas oestradiol benzoate	3-benzoyloxy-17β-hydroxy-oestratriene-1,3,5(10)
oestradiolum oestradiol	3,17β-dihydroxyoestratri-ene-1,3,5(10)
oestronum oestrone	3-hydroxy-17-oxoestratri-ene-1,3,5(10)

[1] This name is to replace "levorphanum" ("levorphan") which had been proposed for the same substance (see list of Proposed International Non-Proprietary Names, *Chronicle of the World Health Organization*, Vol. 7, No. 10, October 1953).

(Proposed International Non-Proprietary Names *contd.*)

pamaquinum pamaquin	8-(4-diethylamino-1-methylbutylamino)-6-methoxyquinoline salt of 2,2′-dihydroxy-1,1′-dinaphthylmethane-3,3′-dicarboxylic acid
pentaquinum pentaquin	8-(5-isopropylamino-pentylamino)-6-methoxyquinoline
pentetrazolum pentetrazol	pentamethylene-1,5-tetrazole
pentolonium pentolonium	1,5-(1,1′-dimethyl-2,2′-dipyrrolidyl)-pentane
pethidinum pethidine	ethyl 1-methyl-4-phenylpiperidyl-4-carboxylate
phenacainum phenacaine	N^1,N^2-bis-(4-ethoxyphenyl)-acetamidine
phenacetinum phenacetin	acetyl-4-phenetidine
phenazonum phenazone	2,3-dimethyl-1-phenyl-5-pyrazolone
phenazopyridinum phenazopyridine	2,6-diamino-3-phenylazopyridine
phenobarbitalum phenobarbital	5-ethyl-5-phenyl-barbituric acid
phenobarbitalum natricum phenobarbital sodium	sodium salt of 5-ethyl-5-phenyl-barbituric acid
phenylhydrargyri boras phenylmercuric borate	equimolecular compound of phenylmercuric borate and phenylmercuric hydroxide
phenytoinum phenytoin	5,5-diphenylhydantoin
pramocainum pramocaine	1-[3-(4-morpholino)-propoxy]-4-butoxybenzene
primidonum primidone	5-ethyl-5-phenyl-4,6-dioxohexahydropyrimidine
progesteronum progesterone	3,20-dioxopregnene-4
proguanilum proguanil	N^1-4-chlorophenyl-N^2-isopropylbiguanide
propoxycainum propoxycaine	2-diethylaminoethyl-4-amino-2-propoxybenzoate
quinisocainum quinisocaine	1-(2-dimethylaminoethoxy)-3-butylisoquinoline
reserpinum reserpine	alkaloid from the roots of *Rauwolfia serpentina* Benth.
riboflavinum riboflavine	6,7-dimethyl-9-(D-1′-ribityl)isoalloxazine
secobarbitalum secobarbital	5-allyl-5-(1-methylbutyl)-barbituric acid
solasulfonum solasulfone	tetrasodium salt of 4,4′-bis-(3-phenyl-1,3-disulfopropylamino)-diphenylsulfone
sorbimacrogoli oleas 100 sorbimacrogol oleate 100	mono ester of oleic acid and tripolyethyleneglycol 100-sorbitan ether
sorbimacrogoli oleas 300 sorbimacrogol oleate 300	mono ester of oleic acid and tripolyethyleneglycol 300-sorbitan ether
stilbamidini isethionas stilbamidine isethionate	1,2-bis-(4-amidinophenyl)-ethene di-(2-hydroxyethane)-sulfonate
succinylsulfathiazolum succinylsulfathiazole	2-(N^4-3-carboxypropionylsulfanilamido)-thiazole
sulfadiazinum sulfadiazine	2-sulfanilamidopyrimidine
sulfadiazinum natricum sulfadiazine sodium	sodium derivative of 2-sulfanilamidopyrimidine
sulfadicramidum sulfadicramide	N^1-(3,3-dimethylacroyl)-sulfanilamide
sulfaguanidinum sulfaguanidine	N^1-amidinosulfanilamide
sulfamerazinum sulfamerazine	2-sulfanilamido-4-methylpyrimidine
sulfamerazinum natricum sulfamerazine sodium	sodium derivative of 2-sulfanilamido-4-methylpyrimidine
sulfanilamidum sulfanilamide	4-aminobenzenesulfonamide
sulfapyroxylinum sulfapyroxyline	N^1-(4-isopropoxybenzoyl)-sulfanilamide
sulfarsphenaminum sulfarsphenamine	disodium 3,3′-diamino-4,4′-dihydroxyarsenobenzene-N,N′-bismethylenebisulfite
sulfathiazolum sulfathiazole	2-sulfanilamidothiazole
suspensio insulini cum zinco (amorphum) insulin zinc suspension (amorphous)	a sterile buffered suspension of the amorphous form of insulin with zinc chloride
suspensio insulini cum zinco (crystallisatum) insulin zinc suspension (crystalline)	a sterile buffered suspension of the crystalline form of insulin with zinc chloride
testosteronum testosterone	17β-hydroxy-3-oxoandrostene
tetrabarbitalum tetrabarbital	5-ethyl-5-(1-ethylbutyl)-barbituric acid
tetracainum tetracaine	2-dimethylaminoethyl-4-butylaminobenzoate
tetracyclinum tetracycline	1-dimethylamino-1,4,6,11,-12,13,14,18-octahydro-2,5,7,11,14-pentahydroxy-4,6-dioxo-11-methylnaphthacene-3-carbonamide
thialbarbitalum thialbarbital	5-allyl-5-(2-cyclohexenyl)-2-thiobarbituric acid
thiaminum thiamine	3-(4-amino-2-methyl-5-pyrimidinylmethyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride
thiopentalum natricum thiopental sodium	mono-sodium salt of 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid
thiotetrabarbitalum thiotetrabarbital	5-ethyl-5-(1-ethylbutyl)-2-thiobarbituric acid
tolonii chloridum tolonium chloride	3-amino-7-dimethylamino-2-methyl-phenazathionium chloride
tosylchloramidum natricum tosylchloramide sodium	sodium toluene-4-sulfonylchloramide
tretaminum tretamine	2,4,6-tri-(ethyleneimino)-s-triazine
tricyclamoli chloridum tricyclamol chloride	(±) 1-(3-cyclohexyl-3-hydroxy-3-phenylpropyl)-1-methylpyrrolidinium chloride
tryparsamidum tryparsamide	sodium N-phenylglycylamide-4-arsonate
viomycinum viomycin	an antibiotic obtained from certain strains of *S. puniceus* or *S. floridae*



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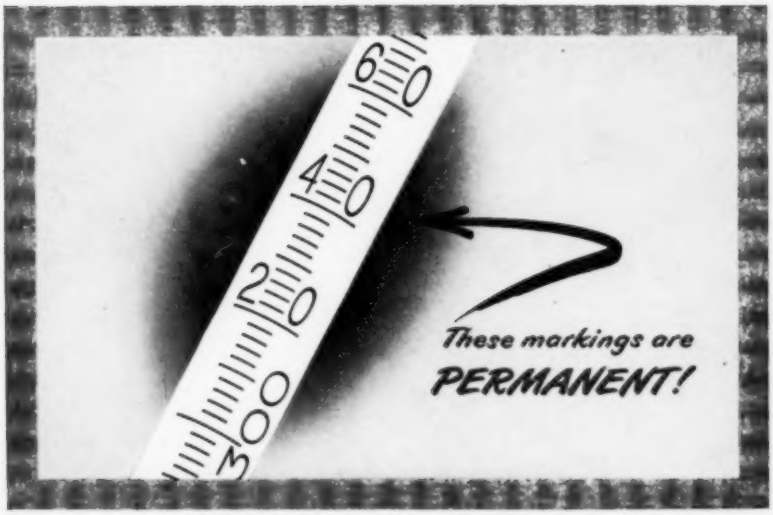

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